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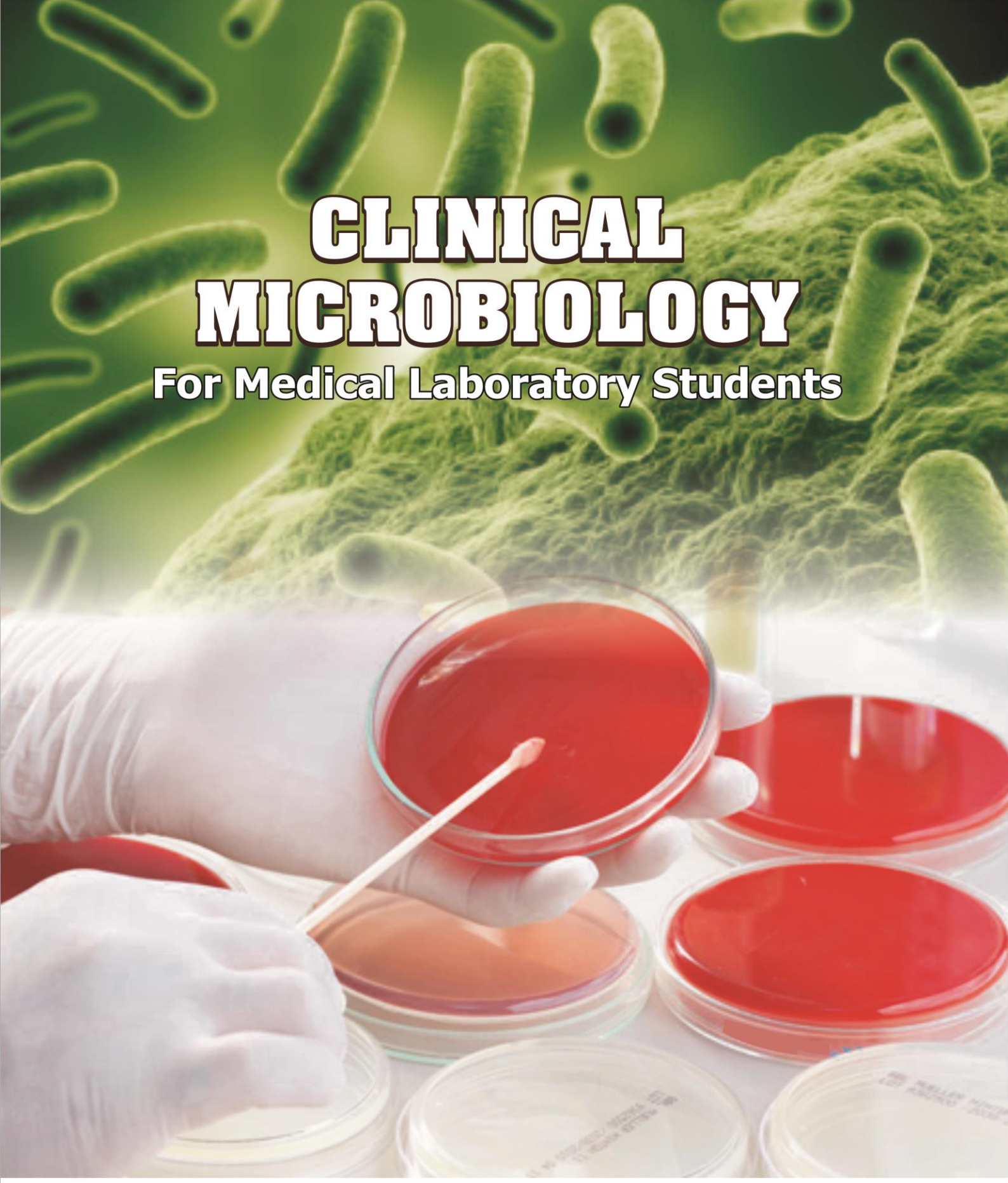

CLINICAL MICROBIOLOGY

For Medical Laboratory Students

Usman Waheed | Anwar Ullah
Asim Ansari | Ihsan Ali

First Edition 2013

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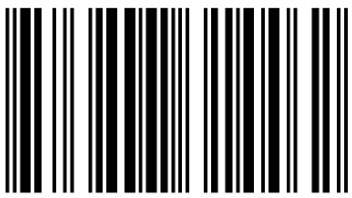

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For Medical Laboratory Students

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Dedicated to

Dr. Khurshid Ahmed

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Preface

Clinical Microbiology is one of the most vital disciplines of pathology and laboratory medicine both at the academic level, where it constitutes a major part of the curriculum of medical laboratory sciences, as well as at the professional level, where it plays a crucial role in the care and management of patients in a modern healthcare system. The need for a well-written compact manual which could not only help the students in their courses of study and training, but also the upcoming laboratory scientist in their day to day work in the microbiology laboratory, especially in a developing country like ours, was being felt since a long time. Concisely written guidelines, covering both the theoretical aspects as well as the practical description of routine methods of microbiological diagnosis, has been the cherished dream of every laboratory professional in our set up. The present manual is a humble effort towards the fulfillment of that dream. Each and every member of this team of medical laboratory professionals has tried his best to present the maximum information in a consolidated form, both practical and theoretical, of their particular area of interest, at one place in this book. Readers will also note that, like our earlier academic ventures, we specifically tried to focus on the syllabi of medical laboratory technology along with the standard operating protocols of the subject in general.

This book is flexibly organized so that topics may be arranged in almost any order. Each chapter has been made as self-contained as possible to achieve this purpose. Some topics are essential to microbiology and have been given more extensive treatment. We have focused on value added features that help a student to check his/her understanding about the subject and support him to start thinking as a member of the health care team. The text is designed to be an effective teaching tool as well. The text is as easy for a student to use as it is easy to read. Readability has been enhanced by using a relatively simple, direct writing style, under many section headings, and in an organized outline format within each chapter. The level of complexity has been carefully set with the target audience in mind. It has really been an exciting moment that nature has once again helped us in bringing out this long-awaited piece of text. We are quite confident that this book will help the students to achieve better grades in exams and will also assist them in striving for excellence in their chosen field.

This task could not be accomplished without the great support of many dearly loved people; here we would like to extend our profound regards to Prof. Hasan Abbas Zaheer, Prof. Haroon Khan, Dr. Mariam Anees, Dr. Aneesa Sultan, Dr. Javed Iqbal Dasti, Patricia Tanabe, Najam Farooq, Ronald Brown, Shafqat Ali, Ishtiaq Ahmed, Ikram Ullah and Zain Tareen. We look forward to the same kind of warm love and support from these well wishers in the upcoming projects and subsequent editions of this book.

Here we are humbly grateful to Dr. Khurshid Ahmed (Scientist Emeritus) who accepted our request to lead in this assignment. We are feeling very honored to be among the team lead by such an influential mentor. We are deeply indebted for his valuable suggestions and scholarly input in the preparation of this manual. Due to his exceptional and matchless contribution to the subject, Microbiology, we would like to dedicate this tiny effort to his dignified personality.

Happy reading.

AUTHORS

Foreword

An oft quoted statistic found in the literature states that 60% to 70% of all medical decisions regarding a patient's diagnosis and treatment, hospital admission and discharge are based on laboratory test results. Although it is difficult to attribute this statistic to a specific reference, one can easily see the importance of accurate and appropriate laboratory testing to patient healthcare and safety. Thus, the qualifications of the laboratory personnel performing laboratory testing are critical if one is to deliver the highest standards of patient care. Credentialing of laboratory personnel certifies professional competency and assures a standard of practice. A properly trained workforce is essential to the delivery of quality patient healthcare.

As the Executive Director of the ASCP Board of Certification (BOC), the oldest and largest certification agency for laboratory professionals, I have the opportunity to meet laboratory professionals from around the world. I met Usman Waheed, one of the authors of this book at Arab Health, Dubai, a large medical conference. Usman Waheed was inquiring about a research article he had submitted to our journal as well as discussing certification and membership opportunities for laboratory professionals. This meeting led to the initiation of an ASCP International (ASCP^{i}) Advisory Board in Pakistan.

The mission of the ASCP Board of Certification is to provide excellence in certification of laboratory professionals on behalf of patients worldwide. The ASCP BOC began international certification of laboratory professionals in 2006. The international advisory boards advocate for the profession as well as promote education and certification. Usman Waheed and Asim Ansari, two of the authors of the *Clinical Microbiology for Medical Laboratory Students* are members of the newly formed Pakistan Advisory Board for ASCP International. Members of the advisory board are chosen for their technical expertise and passion for the profession.

Good resources are essential for training laboratory students and also serve as bench references in the laboratory. There is a tremendous need for a technical handbook that bridges the gap between academic coursework and the clinical testing performed in the laboratory. The *Clinical Microbiology for Medical*

Laboratory Students is such a handbook which serves as a primer to those training in microbiology. It provides the background for the techniques, tests and stains commonly used in the clinical microbiology laboratory. Each laboratory will have its own protocols and procedures. However, a well-trained medical laboratory technologist/technician will come to their place of employment with the core knowledge necessary for them to be successful as a laboratory professional.

Because you are enrolled in a medical technology programme, you have begun a journey towards a rewarding career as a laboratory professional. Take every opportunity to increase your knowledge in the ever changing world of medical laboratory technology. The *Clinical Microbiology for Medical Laboratory Students* will provide you with the essential background necessary to be successful in the microbiology laboratory. Continue to learn and to be curious.

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SECTION-A INTRODUCTION TO MICROBIOLOGY

1. GENERAL BACKGROUND

Microbiology is a branch of medical science which deals with the study of micro-organisms. Micro-organisms are also referred to by their shorter name - microbes. These are very small and cannot be seen by the naked eye, can only be seen with a microscope. These organisms include bacteria, viruses, parasites and fungi. Viruses are non-cellular, live only inside living cells, visible under electron microscope while bacteria are cellular, capable of free living and visible under ordinary microscope. Similarly viruses and fungi are non-motile while parasites and some bacteria are motile. Chlamydia, Rickettsiae and Mycoplasma are among the subgroups. Chlamydia and rickettsiae resemble bacteria in many aspects and are, therefore, traditionally grouped with bacteria. However, unlike bacteria they grow and replicate only inside living cells. Mycoplasma do not require living cells for replication but unlike bacteria, they do not possess a rigid cell wall.

Microbes are literally everywhere. They can be found in the air, soil, water, rocks and bodies of other living organisms. Despite they are undersize, these microbes have an enormous impact on our lives. It has been estimated that there are 5 million trillion, trillion, microbial cells on Earth. Microbes are an incredibly diverse group of organisms and can grow in extreme environments that no other living organisms can tolerate.

Although microbiology is a relatively young science it has an enormous impact on our health and well being. The study of microbiology is very important because of the infectious diseases which have been a threat to human society since the inception of mankind. With very much expanded trade and travel, infectious diseases have crossed borders and oceans spreading at a faster rate resulting in significant mortality, morbidity and economic loss. If the proportion of people living in poverty is to be lastingly reduced, integrated and sustainable measures to reduce infectious diseases are indispensable. Almost all developing countries (including Pakistan) are facing abysmal situation of healthcare as a result of infectious diseases. Studies have revealed that more than 43% of all deaths in developing countries are due to infectious diseases. Many of these

infectious disease- related deaths are caused by infections such as hepatitis, malaria, tuberculosis, HIV/AIDS, diarrheal disease, ARI (acute respiratory infections) and dengue. Besides being pathogenic, microbes are also helpful, e.g. in food production, drugs manufacturing, etc.

The existence of microorganisms was hypothesized for many centuries before their actual discovery. However, it was in 1676, when Anton van Leeuwenhoek examined stagnant rain water with his home-made microscope and saw micro-organisms which he called 'animalcules'. Since then, the science of microbiology has flourished considerably. Now, the science of microbiology is very broad and integrated with many other disciplines such as: Industrial Microbiology, Marine Microbiology, Agricultural Microbiology, Soil Microbiology, Molecular Microbiology, Environmental Microbiology, Medical/Clinical Microbiology, Evolutionary Microbiology and Microbial Genetics. There is considerable overlap between the specific branches of microbiology with each other and with other disciplines. There are many research journals and professional societies dedicated to the field of microbiology. Microbiology is very much a 'hands-on' discipline. In several ways, it is an art as much as a science, and one in which an individual scientist's knowledge, judgment and interpretive skills are crucial.

In Pakistan, Microbiology is regarded as a specialized field of Pathology and Medical Laboratory Technology (MLT) with BS to PhD level academic programmes[1] being offered in addition to Fellowships. As regards the Medical Laboratory Technology, the focus of the study is medical/clinical microbiology where Medical Technologists are entrusted with the task of inspecting the role of micro-organisms in human diseases and try to find ways to prevent and curb these diseases. They isolate, identify and differentiate non-pathogens from those that are involved in a human disease. In practice, the technical personnel need to have knowledge of only a limited number of pathogens and to employ the most sensitive and cost-effective methods to differentiate them from normal body flora in order to achieve meaningful results. They will also find that a large majority, almost two-third, of specimens received in a clinical microbiology laboratory is either negative or yields only normal body flora on culture.

[1] 10 universities offering BS in Microbiology while 7 offer MS in Microbiology

2. SAFETY PRECAUTIONS AND REQUIREMENTS IN MICROBIOLOGY LABORATORY

2.1 SAFETY PRECAUTIONS/GOOD LABORATORY PRACTICES

Almost all procedures performed in the microbiology laboratory involve the use of living micro-organisms; therefore, it is vital to apply aseptic techniques at all times. All micro-organisms should be treated as potential hazards. Some primary safety precautions are:

1. Never place books, coats, etc. on bench tops. Place them at specified area.
2. To prevent contamination from air, close all windows and doors.
3. Clean the bench tops with an appropriate disinfectant at the beginning and ending of laboratory work. One of the best general purpose disinfectant for microbiology laboratory is 10% sodium hypochlorite (bleach).
4. Never take personal items such as combs, cosmetics or handbags into the laboratory.
5. Use liquid detergent for washing hands on entering and leaving the laboratory.
6. Always wear close shoes in the laboratory.
7. Switch off mobile phones in the laboratory.
8. Eating, drinking or smoking is prohibited in the laboratory.
9. Avoid mouth pipetting and always use suitable dispensing tools.
10. Avoid un-necessary movement in the laboratory.
11. Speak quietly to avoid distraction.
12. Cover the spilled or broken cultures immediately and then clean with a disinfectant.
13. Avoid practices which could result in needle stick injury.
14. Cover any open wound with adhesive plasters.
15. All pipettes must have cotton plugs to minimize contamination of pipetting devices.
16. A laboratory coat must always be worn. Ensure it is decontaminated and laundered correctly.
17. Wear protective gloves and when indicated, a face mask.
18. Try to minimize the creation of aerosols.
19. Infectious materials should not be mixed with non-infectious material.

20. Do not allow un-authorized persons to enter the laboratory.
21. It is important that all staff dealing with infectious material should be vaccinated.
22. Report all spills, accidents or potential exposures to infections materials immediately to the head of the laboratory.

2.2 GENERAL REQUIREMENTS OF A MICROBIOLOGY LABORATORY

2.2.1 Equipment

1. Microscope
2. Incubator
3. Autoclave
4. Hot air oven
5. Lovibond comparator
6. Refrigerator
7. pH meter
8. Desiccator (to dry syringes)
9. Wire loop
10. Candle jar
11. Centrifuge
12. Vaccine bath
13. Petri dish
14. Anaerobic jar
15. Thermal cycler (for PCR)
16. Tuberculin syringe (for Mantoux test)
17. Bijou bottle (culture medium holder)
18. BACTEC MB9000 (MB) system

2.2.2 Glassware

1. Glass slides
2. Screw cap tubes
3. Test tubes
4. Flasks
5. Reagent bottles
6. Dropping bottles
7. Petri dishes
8. Pipettes
9. Pasteur pipettes
10. Beakers
11. Cylinders
12. Durham's tubes

2.2.3 Culture Media/Stains

1. Nutrient broth
2. Nutrient agar
3. MacConkey (MAC) agar
4. Blood agar
5. Löwenstein-Jensen media (LJ)
6. Eosin Methylene Blue (EMB) agar
7. SS agar
8. Sabouraud Dextrose Agar (SDA)
9. TCBS agar
10. Tellurite agar

11. Phenylethanol Agar (PEA)
12. Simmons Citrate Agar
13. Spirit Blue Agar
14. Tryptic Soya Agar (TSA)
15. Mannitol salt agar
16. Tryptone soya broth
17. Lactose broth
18. Stuart's transport media
19. Peptone
20. Gelatin
21. Crystal violet powder
22. Safranin
23. Iodine
24. Methylene blue
25. Carbol fuchsin
26. Lacto-phenol blue
27. Basic fuchsin

2.2.4 Chemicals

1. Sodium chloride
2. Potassium hydroxide
3. Potassium iodide
4. Ethanol
5. Phenol
6. Hydrogen per oxide
7. Sodium citrate
8. Urea
9. Glucose
10. Hydrochloric acid
11. Para dimethyl amino benzaldehyde
12. Ether
13. Methyl red
14. Sodium hydroxide
15. Phenol red
16. Tetramethyl para phenylene Diamine-hydrchloride
17. Dipotassium hydrogen phosphate
18. Ammonia
19. Phosphoric acid
20. Citric acid

2.3 POSSIBLE ROUTES OF EXPOSURE TO INFECTIOUS AGENTS IN MICROBIOLOGY LABORATORY

Route	Situation
Ingestion	Mouth pipetting Splashed infectious material Contaminated clothing, devices or fingers Consumed food
Inoculation	Needle stick injury Cuts from sharp objects

Skin and mucous membrane contamination	Splashes into eyes, mouth, nose Spills or splashes on intact or non-intact skin
Inhaled infectious aerosol	Streaking media Flaming or cooling inoculating loop Mixing microbial suspensions by pipette Expelling air from a syringe Withdrawing needle from rubber stopper Separating needle from syringe Centrifuging specimens Mixing instruments such as blenders or shakers Pouring or decanting fluids Opening culture containers Spilling infectious material

3. BIOSAFETY LEVELS

The application of safety precautions to reduce the risk of a laboratory worker to a potentially infectious microorganism is called Biosafety. It also involves limiting the contamination of the laboratory environment and the community. Biosafety can be achieved by various methods and there are four levels. Each level has specific controls for limiting the microorganism and biological agents. Bio-containment is classified by the relative danger to the surrounding atmosphere as biological safety levels.

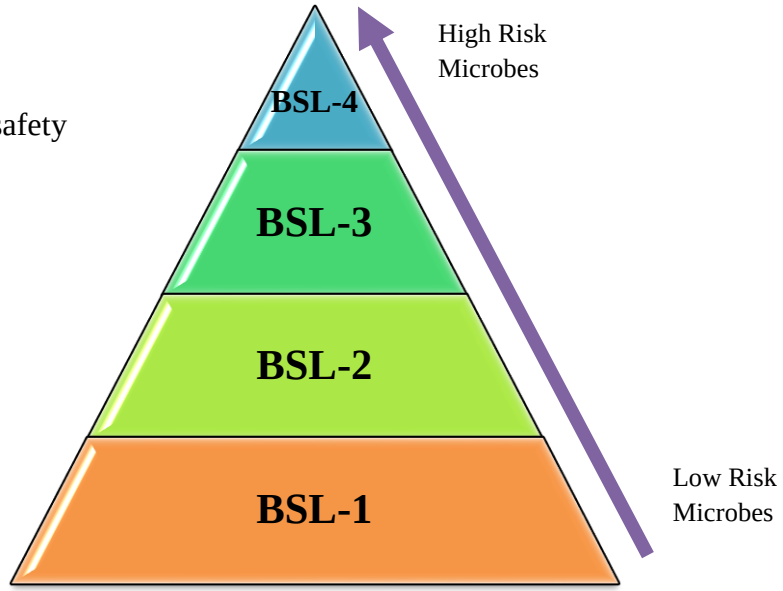


Fig 3.1 Levels of Biosafety

3.1 BIOSAFETY LEVEL 1

This level is suitable for laboratories involving well-characterized agents that present low risk to personnel and the environment. These organisms are highly unlikely to cause infection in healthy laboratory workers, animals or plants, e.g. non-pathogenic Escherichia coli, Agrobacterium radiobacter, Aspergillus niger. An open bench or fumed hood is used for practical work. Decontamination is accomplished by treating with chemical disinfectants or by moist heat autoclaving.

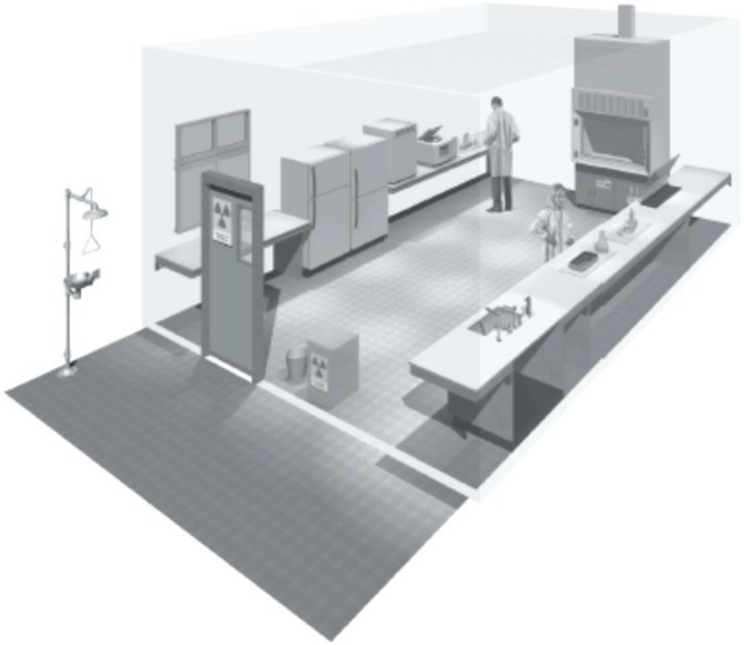


Fig 3.2 **Biosafety Level-1** *Reproduced with permission from WHOs Laboratory Biosafety Manual, 3rd Edition, 2004 ISBN 92 4 154650 6*

3.2 BIOSAFETY LEVEL 2

This group consists of organisms that offer a moderate risk to personnel and the environment. In case, there is any exposure within the laboratory, the risk of transmission is minimal and it hardly ever cause infection that would lead to serious disease. Effective treatment and preventive procedures are available. The microbes require Biosafety Level 2 containment. Examples of BSL-2 organisms are: Mycobacterium, Streptococcus pneumonia, C. difficile, Salmonella, Staphylococcus aureus. Right to use the laboratory is restricted. Biological safety cabinets are required.

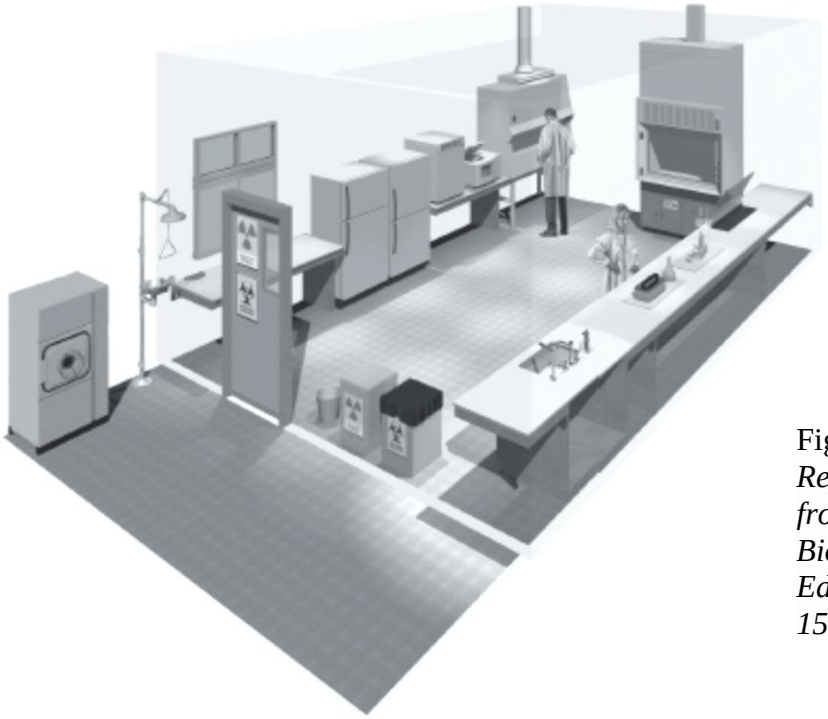


Fig 3.3 **Biosafety Level-2** *Reproduced with permission from WHOs Laboratory Biosafety Manual, 3rd Edition, 2004 ISBN 92 4 154650 6*

3.3 BIOSAFETY LEVEL 3

This level is appropriate when dealing with infectious microbes which may cause serious or potentially fatal disease or can even result in serious economic consequences. These microbes are usually not transmitted by casual contact. The examples include: Mycobacterium tuberculosis, SARS corona virus, L. donovani, West Nile virus and virus of yellow fever. It is essential that laboratory is placed in an isolated area with a double door entry and air flow with inward direction.

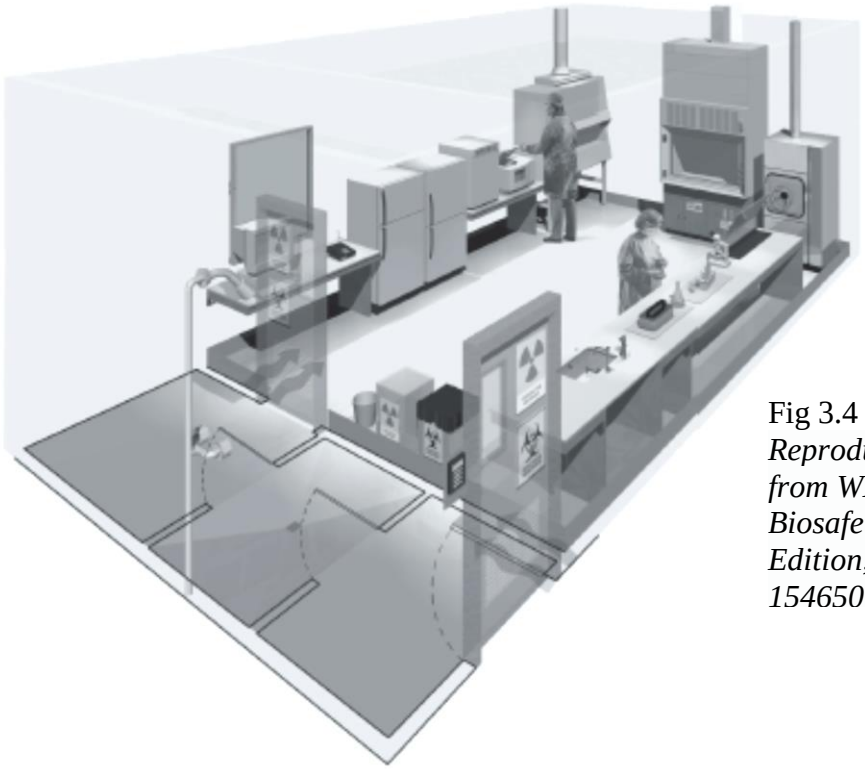


Fig 3.4 **Biosafety Level-3** *Reproduced with permission from WHOs Laboratory Biosafety Manual, 3rd Edition, 2004 ISBN 92 4 154650 6*

3.4 BIOSAFETY LEVEL 4

This level includes those microbial agents which cause deadly diseases and which are usually not treatable. These microbes are spread from one person to another, from animal to human or vice-versa, either directly or indirectly, or by casual contact. The example of these microbes include, Ebola virus, marburg virus, Lassa virus and Crimean-Congo haemorrhagic fever virus. This type of laboratory

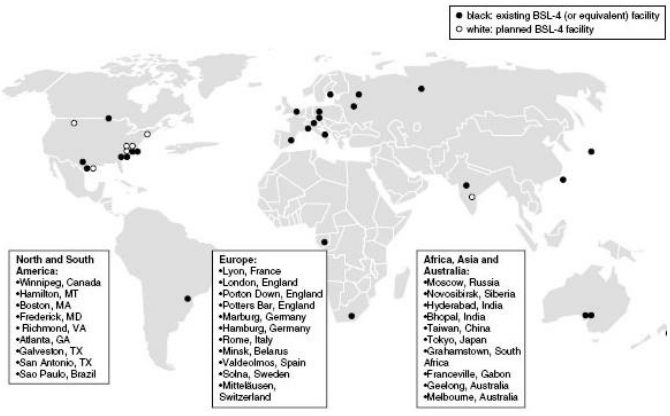


Fig 3.5 BSL-4 Facilities Worldwide

either is located in a separate building or in a controlled area within a building, which is completely isolated from all other areas of the building. The laboratory must have dedicated supply and exhaust air, and in addition vacuum lines and decontamination systems. There are only limited laboratories across the globe as can be seen from the figure.

4. CLASSIFICATION OF MICRO-ORGANISMS ON THE BASIS OF HAZARD

There are micro-organisms which are more hazardous to handle and have more potential to infect laboratory workers than others. Based on this potential, the World Health Organization (WHO) has classified organisms into four Risk Groups.

4.1 RISK GROUP I

This group includes microbes which poses a small risk to the laboratory workers and to the community. This group is less likely to result in causing human disease, e.g. bacteria which spoil food and some common yeasts and moulds.

4.2 RISK GROUP II

The organisms in this group present a moderate risk to the laboratory workers but a low risk to the community. These are able to cause human diseases but not considered a serious hazard. Effective preventive measures (immunization) and treatment (chemotherapy) are available and the risk of transmission in the community is also limited. The organisms in this group are Staphylococci, Streptococci, Clostridia, Vibrios, Enterobacteriaceae, Adenoviruses, Polioviruses, Hepatitis viruses, Toxoplasma and Leishmania, etc.

4.3 RISK GROUP III

The microbes in risk group III present a high risk to the laboratory workers but a low risk to the community. These organisms are not easily transmitted. There are effective vaccines and therapeutic treatment available for risk group III and include Brucella, Mycobacterium tuberculosis, Salmonella typhi, Arboviruses, Plague organisms, Rickettsiae, Chlamydia, etc.

4.4 RISK GROUP IV

The micro-organisms in this group are all viruses. They present a high risk to the laboratory workers and also to the community. Serious diseases can be caused by members of this group and are easily transmissible from one person to another. At present, these are no vaccines or chemotherapeutic materials against these viruses. These viruses include viruses of certain hemorrhagic

fevers (Marburg, Lassa, Ebola, Equine) and other encephalitis viruses. Some Arboviruses are also included in this group.

The laboratory facilities are designated as Basic Laboratory (Risk Group I and II), Containment Laboratory (Risk Group III) and Maximum Containment Laboratory (Risk Group IV). Basic Laboratory falls in the category of BSL 1 and 2, Containment Laboratory in BSL 3 while Maximum Containment Laboratory in BSL 4.

5. INFECTIOUS WASTE DISPOSAL

All laboratories, blood centres and establishments dealing with blood, blood products and body fluids should have appropriate measures in place for infectious waste disposal. The presence of an infectious microbe is essential for a waste to be called Infectious. However, microbial cultures, human blood and blood products, and infectious sharps are believed to be infectious wastes, regardless of whether the presence of infectious agents is verifiable.

Sharps are separated and collected in plastic needle boxes. Sharps include all articles of glass, needles blades, etc. Saturated dressings, plastic blood collection tubes, gauze, and culture plates are segregated and collected in a trash container lined with a red plastic bag. Sharps containers are not filled over 3/4 full and the containers lid is tightly secured at all times. It is mandatory to label and identify rooms and containers used for infectious waste with the words "Infectious Waste" or the international symbol of biohazard.

Fig 5.1 Sharps Container

Fig 5.2 International Biohazard Label/Symbol

Care should be taken that waste containers are not to be accessible to patients and/or the public. Red plastic puncture proof containers which are labeled for sharps, are used for the needles, lancets and syringes. These containers should also include any broken glass. When the incinerator is operational, the infectious waste is put into the incinerator. In case, the incinerator[2] is not operational on a particular day, the infectious waste is stored in a covered secure location until final

Fig 5.3 Infectious Waste Bag

[2] It is a waste treatment procedure that involves the combustion of organic substances contained in waste materials.

disposal. It is must to dispose off infectious waste within 2 days. After Incineration, remove ash and dispose it in an ash pit.

There are some important precautionary measures which need to be followed while handling infectious waste:

- Follow procedures to reduce disease transmission;
- During the handling of waste, wear protective dress;
- Hand washing is mandatory after dealing with waste;
- Do not sort through waste;
- Maintain laboratory very clean and hygienic inside and out;
- Report any injuries urgently to laboratory manager/in charge; and
- Post-Exposure Prevention (PEP) treatment should be done if there is any needle stick injury.

In Pakistan, waste management and biosafety rules[3], guidelines[4] have been developed and published; many hospitals and blood centres dispose their waste improperly because of a lack of proper equipment, necessary consumables and dearth of required training, thereby endangering the environment through contamination with pathogens found in contaminated blood and blood products. This problem can be overcome by ensuring proper waste management implementation and regular trainings of the staff on infection control/waste management guidelines.

[3] Pakistan Biosafety Rules S.R.O 336(I)/2005, under the Pakistan Environmental Protection Act, 1997 (XXXIV of 1997)

[4] National Biosafety Guidelines. Pakistan Environmental Protection Agency. Ministry of Environment, Government of Pakistan. Notification No. F.2(7)95-Bio.

6. STERILIZATION

Sterilization is a process in which every living microorganisms (including bacterial spores) are killed. Sterilization can be done by physical, chemical and physiochemical means. Chemicals employed as sterilizing agents are called chemisterilants. Other important terms associated are Disinfection (killing of many but not all micro-organisms), Decontamination (technique of removing contaminating pathogenic microorganisms from the items by a process of sterilization or disinfection), Antiseptics (chemicals used to kill micro-organisms on the surface of skin and mucous members), Bacteriostasis (a condition where the multiplication of the bacteria is slowed down without killing them) and Bactericidal (chemical that can kill or inactivate bacteria). The microorganisms die at a certain rate which is termed Rate of Killing. The rate of killing depends on concentration of killing agent and the length of time for which it is applied. There are several methods by which sterilization can be done, i.e. Physical [heat (dry and moist), radiations, filtration] and Chemical.

6.1 DRY HEAT

Dry heat works by protein denaturation, oxidative damage and toxic effects of raised levels of electrolytes. This heat is not a powerful bactericidal agent when compared with moist heat owing to its lower power of penetration. The methods of application of dry heat include:

6.1.1 Red Heat

The items including bacteriological loops, straight wires, forceps tips and searing spatulas are sterilized by holding them in Bunsen flame till they become red hot. The article must be kept vertically in the flame until red-hot.

6.1.2 Flaming

This is a method of passing the article over a Bunsen flame, but not heating it to redness. This method does not ensure complete sterilization. The mouth of culture tube is sterilized in this way. Cracking of glassware is occasionally seen. Other articles sterilized by this way are scalpels, flasks, glass slides and cover slips.

6.1.3 Incineration

This is a process of destroying infectious material by burning them in incinerator. Items including soiled dressings, animal carcasses, pathological

material and bedding, etc. should be incinerated. This method results in the loss of the article, hence is suitable only for disposable items.

6.1.4 Hot Air Oven

This method was introduced by Louis Pasteur. It is used for metallic instruments (like forceps, scalpels, scissors), glass wares (such as petri-dishes, pipettes, flasks, all-glass syringes), swabs, oils, grease, petroleum jelly and a few pharmaceutical products. Basically it is applied for substances which can withstand high temperatures and cannot be reliably penetrated by moist heat. A holding period of 1 hour at 160^0C is required for sterilization. It means 1 hour after attaining 160^0C. However, temperature in the range of 180^0C for 2 hours is also used. The efficiency of hot air oven may be checked (quality control) by three different methods (biological, physical, chemical).

Physical: Temperature chart recorder and thermocouple.

Chemical: A special type of tube, Brown's tube 3 is available which requires a temperature of 160^0C for 1 hour to make its colour change. (green spot, colour changes from red to green)

Biological: 10^6 spores of Bacillus subtilis var niger or Clostridium tetani on paper strips are placed inside envelopes and then placed inside the hot air oven. Upon completion of sterilization cycle, the strips are removed and inoculated into thioglycollate broth or cooked meat medium and incubated at 37^oC for 3-5 days. Proper sterilization should kill the spores and there should not be any growth.

6.1.5 Infra red Rays

These rays are generated by an electric element and are then allowed to fall on the object to be sterilized (temperature 180^0C). The items are placed at that temperature for a period of 7.5 minutes. Syringes, metallic instruments are sterilized by this method; however, it is not suitable for larger objects. It needs special equipments and, therefore, is not appropriate in diagnostic laboratory. Efficiency/quality can be checked using Browne's tube No.4 (blue spot).

6.2 MOIST HEAT

In general, the moist heat kills microbes by coagulation and denaturation of proteins. Moist heat is superior to dry heat in action. In addition to coagulation and denaturation of proteins, membrane damage and enzymatic cleavage of DNA may also be involved. Moist heat sterilizes at a low temperature than dry

heat. In moist heat, water helps in the disruption of non-covalent bonds, e.g. H bonds, which hold protein chains together in their secondary and tertiary structures. The methods of application of moist heat include:

6.2.1 Pasteurization

This process is named after Louis Pasteur who discovered that mild heating at 50-60^{0}C would prevent the spoilage of wines and beers. The method was subsequently applied to food and dairy industry. The temperature required is either 63-66^{0}C for 30 minutes (Holder method) or 72^{0}C for 15 seconds (Flash method) followed by rapid cooling at 13^{0}C. For milk, Ultra-High Temperature (UHT), 140^{0}C for 15 seconds and 149^{0}C for 0.5 seconds is used. This technique is appropriate in destroying most of the milk pathogenic organisms, e.g. Salmonella, Mycobacteria, Streptococci, Staphylococci and Brucella. Efficacy/quality is checked by phosphatase test and methylene blue test. Pasteurization may also be used for eating utensils, clothes and bed sheets of patients.

6.2.2 Inspissation

Inspissation is used to disinfect and solidify media which contains serum and egg. Such media are placed in the slopes of an inspissator and heated at 80-85^{o}C for 30 minutes on three successive days. On the 1st day, the vegetative bacteria will die and those spores that germinate by next day are then killed the following day. The method depends on germination of spores in between inspissation. If the spores are not able to germinate, this method cannot be considered sterilization.

6.2.3 Boiling

Boiling in a water bath (100^{0}C) is a traditional method of sterilizing instruments such as scissors, knives syringes, etc. but it may not be able to kill spores. Moist heat at 100^{0}C continuously for 90 minutes is used to sterilize bacteriological culture media. The addition of 2% sodium carbonate to boiling water increases the bactericidal effect.

6.2.4 Tyndallization

A steamer, in which bottles are placed on perforated shelves above boiling water, is frequently used in bacteriological laboratories for sterilizing media which might be damaged by heating above 100^{0}C. The most certain method is the intermittent exposure to free steam at 100^{0}C for 20-30 minutes for three consecutive days and known as tyndallization. It is used, as explained above, for

materials which are destroyed by prolonged heat such as media containing sugars, e.g. TCBS agar.

6.2.5 AUTOCLAVE

In autoclaves, moist heat above 100^0C is achieved by using heat under pressure. Steam under pressure is a very effective sterilizing agent because the temperature is raised above 100^0C, steam penetrates materials such as surgical dressing, theatre gowns very effectively, and a large amount of latent heat is given out when steam condenses to water.

6.2.5.1 Principle

The water boils at 100^0C and at this temperature vapour pressure equals the atmospheric pressure. In an autoclave, the water is boiled in a closed chamber, the pressure inside increases with a corresponding rise in the boiling point of the water. The steam thus formed is superheated, much above 100^0C. Thus in an autoclave, the articles are exposed to moist heat at higher temperatures than 100^0C. At a pressure of 15 psi within the autoclave, the temperature is exact 121^oC. The items are exposed to this temperature for 15 minutes to ensure sterility. However, for the destruction of microbes which cause spongiform encephalopathies (prions), higher temperatures or longer times are applied; 135^oC or 121^oC for at least one hour are recommended. The steam which is superheated in an autoclave condenses on cooler objects and discharges thermal energy as well as moisture. The combined effect of both these is denaturation of microbial proteins. Majority of culture media are sterilized by autoclaving. This destroys the bacterial endospores as well as vegetative cells. The correct temperature and time is vital in autoclaving as over autoclaving can cause change in pH, precipitation and destruction of essential components.

6.2.5.2 Operation

Steam is first introduced into the jacket, which is kept filled throughout the day at a temperature of 121^0C. When the jacket is hot, the load is placed in the chamber and the door is closed and steam is allowed to enter the chamber. The air and condensate start coming out of the discharge channel and when all the cool air is discharged and pure steam starts coming out, a temperature of 121^0C is reached and the steam trap is automatically closed. After this the holding period stars which differs for different articles. At the end of holding period, supply of steam is stopped but to jacket it is maintained. The steam left in the chamber begins to cool by loosing heat and hence the pressure starts falling.

6.2.5.3 Quality Control Testing of Autoclave

The quality of autoclave can be checked by using following physical, chemical and biological control/indicators.

Physical Control: (i) A recording thermometer makes a graphic time record of the temperature changes in the discharge channel and therefore, helps the operator to avoid errors in timing and holding period. (ii) The Thermocouple measurement of Load Temperature is used for finding the heating up time for a given kind of load. A thermocouple is inserted deep inside an article in autoclave chamber. Its leads are carried out under the channel door and connected to the potentiometer. It indicates the temperature inside the test article during autoclaving.

Chemical Control: (i) Brown's control tube 1 contains a red solution which is turned green when heated at 121^0C. (ii) Bowie dick tape is an adhesive tape which is used for articles to be autoclaved. In case the process was successful, dark brown strips appear across the tape. (iii) Succinic acid (whose melting point is 121^oC).

Biological Control: A spore indicator is used. Filter paper strips are impregnated with a sporing organism, such as Bacillus stearothermophilus (10^6). This strip is enclosed in an envelope, and placed in a convenient part of the material undergoing sterilization. The strip must be placed in a position where steam is least likely to penetrate. After removal, strip is cultured in an appropriate medium and incubated for 7 days at 55^0C. No growth indicates effective sterilization.

6.2.5.4 Autoclaving Precautions

- A layer is formed by the air at the bottom of autoclave because air is denser than steam. This result in incomplete sterilization and items below the layer are not sterilized. Therefore, all air should be removed.
- The steam should be saturated and dry.
- Autoclaves must not be overloaded.
- It is important to use correct holding time to avoid under-autoclaving or over-autoclaving.
- An autoclave must be cleaned, maintained and serviced regularly.
- Be careful in opening the autoclave. Open it only when the pressure gauge is showing 'O' and the autoclave is fully vented, otherwise breakage of article take place.

6.2.5.5 Types of Autoclave

The autoclaves can be Simple non-jacketed, Steam jacketed with auto air discharge and High pre-vacuum sterilizer.

6.2.5.6 Factors Affecting Heat Sterilization

- Nature of heat: Dry heat is less effective than moist heat.
- Temperature and time: Temperature and time are inversely proportional. As temperature increases, the time taken decreases.
- Number of microorganisms: If the microbes are more, high temperature for more duration is applied.
- Nature of microorganism: The sensitivity to heat may vary because of species and starin, e.g. spores are heat resistant.
- Presence of organic material: Organic materials such as protein, sugars, oils and fats boost the time required.
- Type of material: If the items are greatly contaminated, high temperature for more duration is applied.

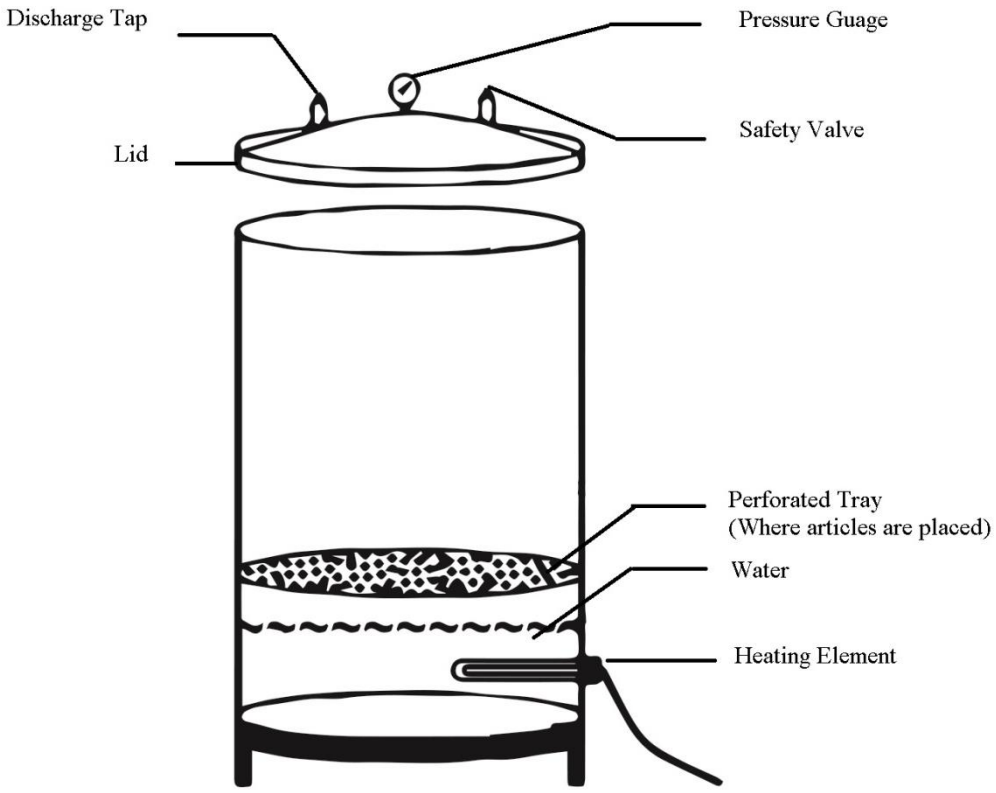


Fig 6.1: Construction of an Autoclave

6.3 RADIATIONS

The two types of radiations used to kill micro-organisms are ionizing and non-ionizing. Non-ionizing rays belong to low energy rays category with reduced penetrative power whereas ionizing rays are high-energy rays with superior penetrative power. Because the radiations do not generate heat, it is called "cold sterilization".

6.3.1 Non-Ionizing Rays (Ultra violet rays)

These rays are longer than the visible light. The microbicidal wavelength of these rays lie in the range of 200-280 nm, with 260 nm as the most effective (the wavelength region of maximum absorption by the purine and pyrimidine bases of DNA). UV rays are produced using a high-pressure mercury vapor lamp. The most significant lesion caused by UV rays is development of thymine-thymine dimers, which eventually inhibits DNA replication, but addition of hydroxyl groups to the bases also occurs. As a result, DNA replication is inhibited and the organism cannot grow. Certain cells have repair mechanisms against VU-induced damage that involve either cleavage of dimmers in the presence of visible light or excision of damaged bases which is not dependent upon visible light. The various categories of microorganisms including bacteria, viruses, yeast, etc. can be deactivated within seconds when exposed to the UV rays. It is, however, critical to note that UV rays do not kills spores. UV rays are considered to be of value in surface disinfection. These are also used to disinfect hospital wards, operation theatres, virus laboratories, corridors, etc. The laboratory application of UV light is limited by its very poor penetrating power. Even a thin glass cover slip is sufficient to protect bacteria on its under surface completely. Very long treatment with UV light is necessary for complete sterilization. Over 90% of M. tuberculosis are killed in 30 minutes by UV light but 15 hours exposure is required to kill all. UV light can cause damage to cornea and skin, therefore, its use is limited in medicine.

6.3.2 Ionizing Rays (X-rays and Gamma rays)

X-rays have higher penetrating power than UV radiation and kill micro-organisms by the production of free radicals, e.g. production of hydroxyl radicals by the hydrolysis of water. These radicals can break covalent bonds in DNA, thereby killing the organism. Spores are resistant to x-rays because they

contain low water. X-rays are used in medicine for sterilization of heat-sensitive items, e.g. surgical gloves and plastic items including syringes.

Gamma rays originate from nuclear disintegration of some radioactive isotopes (Co^{60}, Cs^{137}). Such rays are considered ideal for sterilization of pre-packed disposable plastics (syringes) because of their high penetrating power. A dosage of 2.5 megarads destroys all bacteria, fungi, viruses and spores. It is employed commercially to sterilize disposable petri dishes, plastic syringes, antibiotics, vitamins, hormones, glassware and fabrics. These high-energy radiations damage the nucleic acid of the microbes. The production equipment is both heavy and costly to install and is therefore, more likely to be used by manufacturers than by pathology laboratories.

6.4 FILTRATION

Filtration is the preferred method of sterilizing certain solutions, e.g. those with heat-sensitive components. This process does not destroy microbes, it separates them out. It is used to eliminate microorganisms from heat labile liquids such as serum, antibiotic solutions, sugar solutions, urea solution. The solutions are filtered to make them 'Pyrogen-Free' prior to acutoclaving. Filters act by physically trapping particles larger than the pore size and by retaining somewhat smaller particles via electrostatic attraction of the particles to filers. The filters are:

6.4.1 Diatomaceous Earthenware Filters

These are usually made in the form of a hallow candle. The two best known types are Berkefeld and Mandler. These filters are efficient but fragile and need careful handling. These are prepared in three grades, V (coarse grade), N (intermediate grade) and W (finest grade).

6.4.2 Unglazed Procelain Filters

These filters are first produced by Louis Pasteur and Chamberland by heating a mixture of Quartz sand and kaolin. These are made in the form of a hollow candle, but are also available in the form of discs. These are available in porosity grades from L1 to L13 which is the finest. These are also called chamberland filters.

6.4.3 Seitz Filters

In these filters, the filter paper is a pad made of chrysotile type of Asbestos fibers with other materials to bind them together. The asbestos pads are

available in varying diameters and usually in two grades of porosity, K for clarifying and EK for removing bacteria. The pads are discarded after use so the clearing problem does not occur. For small amounts, Hemming's filter can be used. It consists of a filter pad fitted between two bijou bottles. The bottles are centrifuged, and the liquid to be filtered is thereby forced through the pad from one bijou to the other.

EKS-2 is used for removal of minute organisms from heavily infected liquids PYR is used for removal of pyrogens.

6.4.4 Sintered Glass Filters

They are often presented in the form of disc fused into a glass funnel. Filters of Grade 5 have average pore diameter of 1-1.5 μm. These filters are washed in running water in opposite direction and cleaned with warm concentrated H_2SO_4 and sterilized by autoclaving. It is not effective for Vibrio cholerae.

6.4.5 Membrane Filters

These are thin membranes of nitrocellulose with pore sizes from 0.5μ to downwards. The finest of them will hold back viruses and the coarser of them may be used for bacteria. They have been used in the bacteriological investigation of water since organisms retained on filter will grow if the filter disc is placed on nutrient medium. The newer ones consist of cellulose diacetate. These membranes have a pore diameter ranging from 0.015 μm to 12 μm. These filters have many advantages over other types, e.g. they are relatively tough and, therefore, easy to handle; are sufficiently inexpensive to be used once only; there is minimal retention of solute; and accurate grading of pore sizes over a wide range.

6.4.6 Air Filters

Air can be filtered using HEPA (High Efficiency Particle Air) filters. They are present in biological safety cabinets. HEPA filters have efficiency level of 99.97% for removing particles >0.3 μm in diameter.

6.5 CHEMICAL STERILIZATION

The chemical agents used for sterilization are numerous and called chemisterilants. Chemicals vary greatly in their ability to kill micro-organisms. This variation is measured by comparing the ratio of phenol concentration to the ratio of other agent concentration which is required for killing under similar conditions. This measurement is termed Phenol Coefficient.

They can be classified according to:

(1) Mechanism of action

Disruption of cell membrane (alcohol, detergents, phenol)

Modification of proteins (chlorine, iodine, hydrogen peroxide, ethylene oxide)

Modification of DNA (crystal violet, formaldehyde, ethylene oxide)

(2) Spectrum of activity

High

Intermediate

Low

(3) *Consistency*

Liquid (alcohol, phenol)

Gaseous (ethylene oxide)

7. QUALITY ASSURANCE IN CLINICAL MICROBIOLOGY

Quality Assurance (QA) includes all those activities which are undertaken in a laboratory to guarantee that the test results are of excellent quality. It broadly comprise of controlling the quality of processes at each and every step, e.g. from specimen collection and transport (pre-analytical), processing in the laboratory (analytical) to the reporting and interpretation of results (post-analytical). Every microbiology laboratory should have its own SOP (Standard Operating Procedures) manual. The manual should cover the following aspects of laboratory practice:

- Collection and transport of specimens;
- Registration and documentation;
- Processing: Stepwise details of procedures; staining, microscopy, inoculation on appropriate media, incubation details as regards temperature, atmosphere etc identification tests, susceptibility testing, recording and reporting of results.

SOPs manual should always be available on the bench, be strictly followed at all times and should be periodically revised and updated. SOPs are an integral part of a quality system, as they facilitate consistency in the performance of procedures in accordance with standards.

The Internal QA is defined as the process which covers all steps involved in laboratory processing right from the collection of specimen to the issuance of final report. The term is often used synonymously with Quality Control (QC). Major areas of internal QC in microbiology include the following:

- QC of culture media
- QC of antimicrobial susceptibility testing (AST)
- QC of equipment
- QC of stains and reagents
- QC of antigens and antiseras

Quality control of culture media and antimicrobial susceptibility testing are by far the most critical areas requiring regular and stringent quality control.

7.1 QC OF CULTURE MEDIA

- Always use commercial dehydrated media;
- Order enough to last not more than 6 – 12 months;
- Write date of receipt on every container;
- Store tightly capped in a dark cool place;
- When opened, mark the date of opening on container;
- Discard discoulored bottles.

Preparation

- Follow the manufacturer's instructions strictly;
- Prepare quantities that will be used up in 2 weeks;
- Use a separate room, a separate area in the lab or a safety cabinet with full aseptic precautions.

Storage

- Store in refrigerator;
- Plate media sealed in plastic bag shelf life 4 weeks;
- Media in screw-capped bottles – 3 months;
- Tubes with cotton wool plugs or loose caps 2 – 3 weeks.

Prepared Media

- **pH:** not routinely required when prepared correctly from dehydrated stock.
- **Sterility testing**: In routine, check sterility of all those prepared media in which blood or other supplements were added after autoclaving. Take 2–3% containers from each batch and incubate for 48 hours. The rest is refrigerated and if > 2 colonies are visible per plate, the whole batch is discarded.
- **Performance testing**: Use stock strains for performance testing as follows: Make a suspension of the stock strain to match 0.5 turbidity standard (same as used for Kirby-Bauer susceptibility test). Inoculate one loopful and incubate for the usual length of time. Read in the usual way.
- Use the following stock strains:

Blood agar	S. pyogenes [CO_2 atmosphere]	Shows growth and beta haemolysis
	S. pneumoniae [CO_2]	Growth & alpha haemolysis
MacConkey agar	E. coli	Pink LF colonies
	Proteus	Colourless NLF colonies
	Enterococcus	No growth
SS ager / DCA	E. coli	No growth
	Salmonella / Shigella	Colourless colonies

- Maintain proper record of sterility and performance testing results.

7.2 QC OF ANTIMICROBIAL SUSCEPTIBILITY TESTING

Antimicrobial susceptibility testing is one of the most critical areas of diagnostic microbiology and, therefore, requires strict quality control. Following steps require particular attention:

- Sensitivity medium (Mueller-Hinton agar);
- Antibiotic discs;
- Turbidity standard;
- Density of inoculums;
- Standard control strains;
- Reading of zone sizes of sample; and
- Comparison with control strains.

Following control strains (if available) should be tested along with the test organisms weekly or at least fortnightly.

E. coli	ATCC 25922
Staph. aureus	ATCC 25923
Pseudomonas aeruginosa	ATCC 27853

1. Mueller-Hinton agar should be poured to a depth of about 4 mm – a 90 mm plate requires 20 – 25 ml of molten agar. It is crucial not to overheat the medium. The surface of the medium should be dry before streaking. Prepared and dried plates can be stored in sealed plastic bags for up to 2 weeks in the refrigerator.
2. Commercially available discs with appropriate diameter (6.35 mm) and potency should be used. A maximum of 7 discs can be placed per plate; 6 at

the periphery, about 15 mm from the edge, and 1 in the centre. The range of locally available antibiotics, cost and local prescribing policies will govern choice of antibiotics for susceptibility testing. The range of first choice drugs should be limited to a basic set of inexpensive antibiotics.

3. Zone sizes should preferably be measured using a caliper or a ruler.

Quality Assurance in AST (Antibiotic Susceptibility Testing)

- A correct content of antibiotic per disc is used and also antibiotic discs which are 6.35 mm in diameter.
- The supply of antimicrobial discs is stocked at -20^0C.
- Mueller-Hinton medium is used for antibiotic sensitivity determination.
- Suitable control cultures are used.
- Place the antibiotic discs at room temperature for 1 hour before use.
- Before reporting, incubate the sensitivity plates at 35-37^{o}C for 16-18 hours.
- Use inoculum size that produces near confluent growth (turbidity standard 0.5)
- Make sure that an even contact of the antibiotic disc with the inoculated medium is made.
- Determine the zone sizes correctly; interpret by referring to standard charts.
- Maintain regular record of AST QC results.

7.3 QC OF EQUIPMENT

All laboratory equipment should be of good quality. It should be properly looked after, maintained and serviced at regular intervals. Major items of equipment which need to be checked regularly include: Autoclave, hot air oven, incubator, refrigerator, water bath, centrifuge and microscope.

7.4 QC OF STAINS AND REAGENTS

Test the stains using appropriate control organisms:

- When a new batch is prepared;
- Every week;
- Discard when manufacturer's expiry date is reached;
- Discard if visible signs of deterioration appear e.g. turbidity, precipitation, discolouration or when the stained slide shows debris.

7.5 QC OF ANTIGENS AND ANTISERA

- Store at recommended temperatures;
- Follow manufacturer's instructions;
- Use before expiry;
- Avoid repeated freezing and thawing; divide into small aliquots.

SECTION-B BACTERIOLOGY

8. BACTERIA

A bacterium is a prokaryotic (simple structure) unicellular organism which is nucleated and has no membrane bounded organelles, capable of free living and divide by binary fission. The size varies and ranges from 0.2 to 5 μm. The smallest bacteria equal the size of the largest virus (poxviruses). The study of bacteria is called Bacteriology. The bacterial cell mainly consists of cell wall, cell membrane, cytoplasm and genetic material, etc. Some bacteria show surface features which are external to the cell wall, e.g. capsule, pilli and flagella but are less common.

Bacteria are given a binomial nomenclature. First part of the name indicates the genus, which is the main group or class to which the organism belongs, and is written with a capital first letter. The second part indicates the species within the genus with all letters written in lower case. Taking the example of Staphylococcus aureus, *Staphylococcus* is the genus and *aureus* is the species. A genus usually consists of a number of species.

A bacterium is called pathogenic if it is capable of causing diseases. The term normal flora is used for bacteria which are residing on an individual permanently and not harmful. The viruses and parasites are not regarded as the members of the normal flora. The members of normal flora have a major role both in maintenance of health and in the causation of disease (only in immune compromised individuals). It has been estimated that the average person is colonized by 200 trillion bacteria, comprising at least 1,000 different species. Taking an example, our intestines contain about 100 trillion bacteria and collectively they make up 60% of the dry weight of faeces. These intestinal bacteria play an essential role in helping us to digest food, they provide us with essential vitamins such as vitamin K and biotin and they help to prevent the growth of harmful pathogenic bacteria. The normal flora can be Opportunists (cause disease if opportunity arises), Commensals (provide protective host defense mechanism) and Symbionts (provide nutritional function).

The division between a pathogen and commensal (normal flora) is often relative and not absolute. An organism that is commensal at one site may behave as a

pathogen if it migrates to another site in the same host. A common example is E. coli, which is a common commensal in GIT but is a serious pathogen when infects the urinary tract. There are four sites in the body which harbor a variety of microorganisms without causing any ill effects to the host. These sites include:

Mouth and Upper Respiratory Tract

- Streptococcus viridians
- Diphtheroids
- Neisseria species
- Bacteroides and other anaerobes

Skin

- Staphylococcus epidermidis
- Diphtheroids

GIT

- Bacteroides
- E. coli
- Proteus
- Enterococcus
- Clostridia

Urogenital Area

- Lactobacilli
- Coliforms
- Staphylococcus – coagulase negative

8.1 CLASSIFICATION OF BACTERIA

8.1.1 ON THE BASIS OF ENERGY SOURCES

8.1.1.1 Phototrophs

Some bacteria, found in water and soils obtain energy from sunlight through the agency of pigments.

8.1.1.2 Chemolithotrophs

These bacteria obtain energy for growth from the oxidation of inorganic compounds.

8.1.1.3 Chemoraganotrophs

These bacteria obtain energy for growth from the oxidation or fermentation of organic compounds. All bacteria of medical importance fall into this last category, or into the even more extreme one, the paratrophs.

8.1.1.4 Paratrophs

These bacteria obtain their energy from the metabolism of the host cell and include viruses, rickettsiae and some bacteria.

8.1.2 ON THE BASIS OF CARBON SOURCES

8.1.2.1 Autotrophs

These bacteria (mostly the phototrophs and chemolithotrophs) are able to grow with CO_2 as the sole carbon source.

8.1.2.2 Heterotrophs

These bacteria require a supply of organic carbon molecules.

[Viruses are hypotrophs as they depend solely on enzymatic apparatus of the host cell for replication]

8.1.3 ON THE BASIS OF TEMPERATUE REQUIRMENTS

8.1.3.1 Thermophiles

These bacteria show optimal growth activity at 55-75^{o}C.

8.1.3.2 Mesophiles

These bacteria show optimal growth activity at 30-45^{o}C. Most of the clinically important organisms belong to this group.

8.1.3.3 Psychrophiles

These bacteria show optimal growth activity at 15-18^{o}C.

8.1.4 ON THE BASIS OF ATMOSPHERIC REQUIREMENTS

8.1.4.1 Obligatory (Strict) Aerobes

These bacteria require oxygen to survive, e.g. Mycobacterium tuberculosis and Pseudomonas aeruginosa.

8.1.4.2 Microaerophilic Organisms

These bacteria also require oxygen but at comparatively low levels (-5% O_2), e.g. Campylobacter jejuni.

8.1.4.3 Obligatory (Strict) Anaerobes

These bacteria require absence of oxygen to survive, e.g. Clostridium species, Actinomyces.

8.1.4.4 Facultative Anaerobes

These can survive with or without oxygen (have aerobic and anaerobic respiration), e.g. Streptococci and Enterobacteriaceae. Overwhelmingly, majority of pathogenic bacteria encountered in clinical microbiology laboratory belong to this category.

8.1.4.5 Carboxyphilic Organisms

These require a small amount of CO_2 (carbon dioxide), e.g. Neisseria meningitidis.

8.1.5 ON THE BASIS OF MORPHOLOGY

8.1.5.1 Cocci

These are round or oval bacteria measuring about 0.5 – 1 µm in diameter. During multiplication, cocci can be seen in pairs (Meningococci and Gonococci), chains (Streptococci) and clusters (Staphylococci).

8.1.5.2 Rods (Bacilli)

These are bacteria (1-10 um in length) which appear as stick-like having rounded, tapered, square or swollen ends. During multiplication, rods may appear in chains (Streptobacilli), branching chains (Lacobacilli) or mass together (Mycobacterium leprae). These rods can also be seen attached at various angles as in case of Clostridium diphtheriae

8.1.5.3 Vibrios

These are small slightly curved rods, measuring 3-4 µm in length. These are motile and have a flagellum at one end, e.g. Vibrio cholera.

8.1.5.4 Spirilla

These are coiled bacteria with 3-4 µm length. These are also motile and have a flagellum at both ends, e.g. Spirillum minus.

8.1.5.5 Spirochaetes

These are also coiled and motile bacteria. They may be Treponemes (thin delicate coils), Borreliae (irregular large open coils) and Leptospires (thin tightly packed coils).

8.1.5.6 Pleomorphic

These bacteria are variable in shape.

8.2 STRUCTURE OF BACTERIA

The bacterial cell mainly consists of cell wall, cell membrane, cytoplasm and genetic material, etc.

8.2.1 Cell Wall

The outer layer of the bacteria is called Cell Wall. Only Mycoplasma species lack a cell wall and are bounded by a cell membrane. The structure, chemical composition and thickness of the cell wall vary in gram negative and gram positive bacteria.

- The layer of peptidoglycan is thicker in gram positive bacteria.
- Gram positive have teichoic acid while gram negative do not.
- Gram negative have outer layer of lipopolysaccharide, lipoprotein and phospholipids.
- Mycobacteria, e.g. (M. tuberculosis), have a unique cell wall and are called acid fast because they resist decolourization with acid alcohol after being stained with carbol fuchsin. This property is attained due to high concentration of cell wall lipid called mycolic acid.

8.2.2 Cytoplasm

The cytoplasm contains amorphous matrix (containing ribosomes, nutrient granules, metabolites and plasmids) and an internal nucleoid region made up of DNA. Bacterial ribosome is the location of protein synthesis. It is 70s in size with 50s and 30s subunits. Plasmid is an extra-chromosomal, double stranded, circular DNA molecule which is capable of replicating independent of the bacterial chromosomes. Transposons are jumping genes that move readily from one side to another site, either with in or between the DNAs of bacteria, plasmids and bacteriophages.

8.2.3 Capsule

It is located outside bacteria and is a gelatinous layer wrapping the entire bacteria. It consists of polysaccharide, except in the anthrax bacillus which has a capsule of polymerized D-glutamic acid. They act as virulence factor by protecting against phagocytosis and also play a role in adherence of bacteria to human tissue.

8.2.4 Flagella and Pilli

These are long whip like appendages that shift the bacteria toward nutrients and other attractants by a process called chemotaxis. Pilli are hair like structure that extends from the cell surface. Pilli are different in appearance than flagella and are shorter and straighter. They mediate attachments of bacteria to specific receptors on the human cell surface.

8.2.5 Glycocalyx

It is a polysaccharide covering secreted by many bacteria and helps the bacteria to adhere firmly to various structures.

8.2.6 Spores

Spores are highly resistant in nature and are developed in reaction to adverse environments. They provide resistance to dehydration, heat and chemicals. This resistance is due to dipicollinic acids and a calcium ion chelator found only in spores.

8.2.7 Exotoxin

These are produced by both gram positive and gram negative bacteria, are polypeptides in nature, highly toxic but relatively unstable.

8.2.8 Endotoxin

These are specifically found in gram negative bacteria and are lipopolysaccharide (LPS) in nature. The stability is more when compared with exotoxin.

9. BACTERIAL STAINING TECHNIQUES

There are several staining techniques performed in a microbiology laboratory to colour bacterial cells and increase their contrast (bacteria are too small and transparent) so they can be seen with a microscope easily.

9.1 GRAM STAINING TECHNIQUE

9.1.1 Principle

This stain was originally described by Christian Gram in 1884. The mechanism of Gram staining is not fully understood. Gram positive bacteria stain with crystal violet and are not decolourized with acetone, while Gram negative bacteria are decolourized with acetone and hence take up the colour of counter stain. The difference in staining is due to the difference in the cell wall structure. Gram positive bacteria have thick layer of peptidoglycan in their wall while Gram negative have thin layer. This may be the cause of retention of crystal violet in the Gram positive bacteria.

9.1.2 Requirements

- Crystal violet stain
- Lugol's iodine
- Acetone-alcohol decolourizer
- Neutral Red
- Staining rack
- Glass slide
- Microscope
- Oil immersion

9.1.3 Procedure

1. A smear is prepared on a glass slide and air dried.
2. It is fixed by rapidly passing the slide, three times through the flame of a spirit lamp.
3. In case of N. Meningitidis and N. gonorrhoeae, fix in methanol for 2 minutes.
4. The fixed smear is covered with the stain (crystal violet) for 1 minute.
5. The stain is washed with water and smear is covered with Iodine solution for 1 minute.

6. Iodine is washed off with water and smear is decolourized with acetone alcohol for a few seconds.
7. Immediately wash and cover with another stain, neutral red for 2 minutes.
8. Wash the stain with water and place in a draining rack to air-dry the smear.
9. Place cover slip and observe the smear microscopically, first with 40x objective to check the staining and to see the distribution of material. This is followed by examining with the oil immersion lens to look for bacteria and cells. It is important that condenser iris is fully open when using oil immersion lens.

9.1.4 Results

Gram positive bacteria	Dark Purple
Gram negative bacteria	Red
Yeast cells	Dark Purple
Epithelial cells	Pale Red
Nuclei of pus cells	Red

9.1.5 Quality Control

To check the efficacy of staining solution and procedure, known Gram positive (Staph. aureus) and Gram negative (E. coli) organisms should be stained on the same slid of test organisms.

9.1.6 Reporting

Following information is reported:

- No. of bacteria present (numerous, moderate, few, or scanty);
- Reaction of the bacteria (Gram positive or Gram negative);
- Morphology of bacteria (cocci, diplococci, streptococci, rods, or coccobacilli);
- Presence of pus cells;
- Number of pus cells;
- Presence and number of epithelial cells and yeast cells.

9.2 ZIEHL – NEELSEN STAINING

The ziehl – neelsen straining technique is used to stain Mycobacteria. These organisms do not stain well by the Gram staining technique.

9.2.1 Principle

Mycobacteria are stained with carbol function which consists of a strong basic dye called basic fuchsine combined with phenol. After staining, an acid decolourizing solution is applied which removes the red dye from the background cells, tissue fibers and any organism in the smear except those that are acid fast. The bacilli which retain the carbol fuchsin are referred to as AFB (Acid Fast Bacilli). Following deodorization, the smear is counterstained with malachite green or methylene blue which stains the background material and provides a good contrast against which the red AFB can be seen. It has been estimated that it takes 50,000 – 10,000 tubercle bacilli/ml of sputum to detect the organisms microscopically in a ZN stained preparation.

9.2.2 Requirements

- Carbol fuchsin stain
- Acid Alcohol 3%
- Malachite green 5g/l
- Staining rack
- Glass slide
- Microscope
- Oil immersion

9.2.3 Procedure

1. A smear is prepared on a glass slide and air dried.
2. The smear is fixed with alcohol by covering the slide with 70% ethanol for 2 minutes.
3. The carbol fuchsin stain is then placed over the fixed smear.
4. The slide is heated until vapour just begins to rise (60^{o}C). This heated stain is allowed to cover the smear for 5 minutes.
5. The stain is washed in running tap water.
6. Cover with 3% acid alcohol for 5 minutes and rinse in water.
7. Wash well with clean water.
8. Cover with the stain malachite green for 1-2 minutes.

9. Wash off the stain with clean water and place in a draining rack to air-dry the smear.
10. Place cover slip and observe the smear microscopically, first with 40x objective to check the staining and to see the distribution of material. This is followed by examining with the oil immersion lens to look for acid fast bacteria. It is important that condenser iris is fully open when using oil immersion lens.

9.2.4 Results

AFB	Red, straight or curved rods
Cells	Green
Background material	Green

9.2.5 Quality Control

To check the efficacy of staining solution and procedure, two sputum smears of known high and low AFB positivity should be stained with the routine smear.

9.2.6 Reporting

If acid fast bacilli are present, report the number as follows;

More than	10 AFB/field	+++
	1-10 AFB/field	++
	10-100 AFB/100 fields	+
	1-9 AFB/100 fields	report the exact number

If no acid fast bacilli are present, report as Not Seen. Do not report Negative because the organisms may be present but not seen.

9.3 ALBERT STAINING TECHNIQUE

It is used to demonstrate metachromatic granules found in Corynebacterium diphtheriae. This bacterium is responsible for the disease diphtheria.

9.3.1 Principle

The granules are called metachromatic granules because it exhibits the property of metachromasia, wherein the granules appear in a colour other than the colour used for staining. When stained with Albert's stain, the bacteria stains green whereas the granules stain bluish black.

9.3.2 Requirements

- Toluidine blue – malachite green stain
- Albert's iodine
- Staining rack
- Glass slide
- Microscope
- Oil immersion

9.3.3 Procedure

1. A smear is prepared on a glass slide and air dried.
2. The smear is fixed with alcohol and covered with the stain toluidine blue-malachite green for 3 minutes.
3. Wash in running tap water.
4. Tip off all the water cover the smear with Albert's iodine for 1 minute.
5. Wash in running tap water.
6. Wash off the stain with clean water and place in a draining rack to air-dry the smear.
7. Place cover slip and observe the smear microscopically, first with 40x objective to check the staining and to see the distribution of material. This is followed by examining with the oil immersion lens to look for bacteria and metachromatic granules. It is important that condenser iris is fully open when using oil immersion lens.

9.3.4 Results

Bacterial cells	Green
Metachromatic granules	Green-Black

9.3.5 Quality Control

Known positive samples must always be used to check the efficacy of staining solution and procedure.

9.3.6 Reporting

The report should include the number of bacterial cells present and colour of granules and bacterial cell.

9.4 HISS STAINING TECHNIQUE

This technique, also called as Capsule Staining Technique, is used for showing the presence of capsules around bacteria.

9.4.1 Principle

The capsule, also called glycocalyx, is a gelatinous outer most layer that is secreted by the bacteria and remains stuck to it. The composition of the capsules may be polysaccharide, glycoproteins or polypeptides depending on the organism. The Capsule or Hiss Staining Method will demonstrate the presence of capsule and assist in identification of bacteria with capsule.

9.4.2 Requirements

- Crystal violet stain
- Copper sulfate
- Staining rack
- Glass slide
- Microscope
- Oil immersion

9.4.3 Procedure

1. A smear is prepared on a glass slide and air dried.
2. The smear is fixed with alcohol and covered with the stain crystal violet stain. It is then heated until vapor just beings to rise. Let the stain on smear for 1 minute.
3. Wash in running tap water and cover with copper sulfate solution.
4. Wash off the stain with clean water and place in a draining rack to air-dry the smear.
5. Place cover slip and observe the smear microscopically, first with 40x objective to check the staining and to see the distribution of material. This is followed by examining with the oil immersion lens to look for bacteria with capsules. It is important that condenser iris is fully open when using oil immersion lens.

9.4.4 Results

Bacterial cell	Dark Purple
Capsule outline	Pale Blue

9.4.5 Quality Control

Known positive samples must always be used to check the efficacy of staining solution and procedure.

9.4.6 Reporting

Report as presence or absence of capsulated bacteria. Examples of capsulated organisms are:

- Streptococcus pneumonia
- Klebsiella
- Haemophilus influenzae

9.5 SPORE STAINING TECHNIQUE

9.5.1 Principle

The spore wall is relatively impermeable, but dyes can be made to penetrate it by heating the preparation. The same impermeability then serves to prevent decolourization with decolourizing agents.

9.5.2 Requirements

- Carbol fuchsin stain (as in ZN stain)
- 0.5% sulphuric acid
- 1% methylene blue
- 5% safranin

9.5.3 Procedure

1. A smear is prepared on a glass slide, air dried and fixed.
2. Stain with carbol fuchsin for 3-5 min with the help of heat until steam just begins to rise. (similar to ZN staining procedure)
3. Wash in water and decolourize with 0.5% sulfuric acid.
4. Wash with water and counter stain with 1% methylene blue for 3 minutes.
5. Wash in water, blot and dry.
6. Place cover slip and observe the smear microscopically. It is important that condenser iris is fully open when using oil immersion lens.

9.5.4 Results

Spores	Red
Protoplasm	Blue

9.5.5 Quality Control

Known positive samples must always be used to check the efficacy of staining solution and procedure.

9.5.6 Reporting

Report as presence or absence of Spore forming bacteria and specify the position of spores whether terminal, sub-terminal or central. Also report number of spores per bacteria. Examples of spore forming organisms are:

- Bacillus species (B. anthracis)
- Clostridia

10. BIOCHEMICAL TESTS FOR BACTERIA

Various Biochemical tests are used for the identification of bacteria.

10.1 OXIDASE TEST

This test helps in the identification of bacteria which produce the enzyme oxidase (Pseudomonas, Neisseria, and Pasteurella species).

10.1.1 Principle

A filter paper piece is mixed with a few drops of oxidase reagent. The test organism is then smeared on the filter paper. The phenylene diamine in the oxidase reagent is oxidized to deep purple colour if the test organism is oxidase positive.

10.1.2 Requirements

- Oxidase reagent (tetramethyl-p-phenylenediamine dihydrochloride)
- Filter paper
- Petri dish
- Glass rod

10.1.3 Procedure

1. The oxidase reagent (2-3 drops) is placed on a piece of filter paper.
2. With the help of a glass rod (sterile), remove a colony of test organism and smear it on the filter paper.
3. Observe for the formation of blue purple colour within 10-15 seconds.

10.1.4 Results

Blue purple colour (in 10 seconds)	Positive
No blue purple colour	Negative

10.1.5 Quality Control

P. aeruginosa is used as positive oxidase control whereas E. coli is used as negative control.

10.2 CATALASE TEST

This test is used to differentiate those bacteria that produce the enzyme catalase, e.g. differentiating staphylococci from streptococci.

10.2.1 Principle

Catalase works as a catalyst in the breakdown of hydrogen peroxide to oxygen and water. The test organism is brought into contact with hydrogen peroxide and bubbles of oxygen are seen if the test organism produces the enzyme catalase.

10.2.2 Requirements

- Hydrogen peroxide 3%
- Test tube
- Glass rod

10.2.3 Procedure

1. Hydrogen peroxide is placed in a test tube.
2. Using a glass rod (sterile) remove a colony of the test organism and immerse it in the hydrogen peroxide solution.
3. Observe for the release of bubbling which indicates presence of oxygen.

10.2.4 Results

Bubbling	Positive
No bubbling	Negative

10.2.5 Quality Control

Positive catalase control is Staphylococcus species while negative catalase control is Streptococcus.

10.3 COAGULASE TEST

This test is used to differentiate Staph. aureus which produces the enzyme coagulase, from other Staphylococcus species which do not produce coagulase.

10.3.1 Principle

Coagulase enzyme acts on plasma and clot it by converting fibrinogen to fibrin. There are two types of coagulase enzyme formed by most strains of Staph. aureus which are Free coagulase (converts fibrinogen to fibrin by activating a coagulase reacting factor present in plasma, and is identified by the appearance of fibrin clot in the test tube) and Bound coagulase (which converts fibrinogen directly to fibrin without requiring a coagulase reacting factor).

10.3.2 Requirements

- Plasma sample (in EDTA)
- Glass slide
- Test tube
- Normal saline

10.3.3 Procedure

SLIDE TEST

1. Put a drop of normal saline on each end of a glass slide.
2. Mix a colony of test organism in each of the drops to prepare two thick suspensions.
3. Place a drop of plasma to one of the suspensions. The other suspension serves as negative control.
4. Mix gently and observe for clumping in next 10 seconds.

TUBE TEST

1. Using normal saline, plasma is diluted 1 in 10.
2. Three tubes are taken and labeled as test, positive control and negative control.
3. Place 0.5 ml of the diluted plasma in all tubes.
4. Put 5 drops of the test organism culture in the tube labeled as Test.
5. Put 5 drops of Staph. aureus culture in the tube labeled as Positive Control. Put 5 drops of sterile broth in the tube labeled as Negative Control.
6. Mix thoroughly and incubate at 35-37^{0}C. Observe for clotting after 60 minutes.

7. If no clotting has occurred, examine at 30 minute intervals for up to 6 hours.

10.3.4 Results

Slide Test

Clumping within 10 seconds	Staph. aureus
No clumping within 10 seconds	No free coagulase produced

Tube Test

Fibrin clot	Staph. aureus
No fibrin clot	No free coagulase produced

10.3.5 Quality Control

Known positive samples (Staph. aureus) are used to verify the result.

10.4 DNAse TEST

This test is used to differentiate Staph. aureus which produces the enzyme DNAse from other staphylococci which are not able to produce DNAse. The test is of value if coagulase test cannot be performed due to non-availability of plasma or even in cases when one cannot interpret the results of coagulase.

10.4.1 Principle

DNAse enzyme causes hydrolysis of DNA. The test organism is cultured on a DNA containing culture media. After incubating overnight, the colonies are tested to check for DNAse production by flooding the plate with a weak HCl solution. The HCl precipitates unhydrolyzed DNA. The colonies producing DNAse are surrounded by clear areas indicating DNA hydrolysis.

10.4.2 Requirements

- DNA agar plate
- HCI 1 mol/l
- Sterile wire loop

10.4.3 Procedure

1. Divide a DNA agar plate into required number of strips by marking the underside of the plate.
2. Using a sterile loop, spot-inoculate the test and control organisms.
3. Incubate the plate at 37^{o}C overnight.
4. Cover the surface of the plate with HCI solution and tip off the excess acid.
5. After adding HCl, observe for clearing around colonies in next 5 minutes.

10.4.4 Results

Clearing around colonies	DNAse positive strain
No clearing around colonies	DNAse negative strain

10.4.5 Quality Control

Known positive samples (Staph. aureus) and negative samples (Staph. epidermidis) are used to verify the result.

10.5 VOGES-PROSKAUER TEST

This test is occasionally used to assist in the differentiation of Enterobacteriaceae.

10.5.1 Principle

The test organism is cultured in glucose phosphate peptone water for 48 hours. Sodium hydroxide and a small amount of creatine are then added. The acetoin produced from fermentation of glucose is oxidized into diacetyl under alkaline conditions and exposure to air. The acetoin develops a pink compound with creatine.

10.5.2 Requirements

- Glucose phosphate peptone water
- Sodium hydroxide solution
- Creatine powder
- Incubator

10.5.3 Procedure

1. Inoculate 2 ml of sterile glucose phosphate peptone water with test organism.
2. Incubate at 37^0C for 48 hours.
3. Add a very small amount of creatine and mix.
4. Add 3 ml NaOH and shake well.
5. Remove bottle cap, leave for 1 hour at room temperature.
6. Look for the development of pink colour.

10.5.4 Results

Pink-red colour	Positive
No pink-red colour	Negative

10.5.5 Quality Control

Known positive samples (K. pneumoniae) and negative samples (E. coli) are used to verify the result.

10.6 INDOLE TEST

This test is used to test for indole production which is helpful for identifying enterobacteriaceae (differentiating lactose fermenting colonies in MacConkey agar). E. coli is indole positive while other enterobacteriaceae species are negative.

10.6.1 Principle

The bacteria which release the enzyme tryptophanase are able to convert the amino acid tryptophan to by-products including indole. When indole is combined with Kovac's reagent or Ehrlich's reagent (containing para dimethylaminobenzaldehyde), the solution turns from yellow to cherry red.

10.6.2 Requirements

- Kovac's Reagent
- Test organism

10.6.3 Procedure

1. Inoculate tryptone or peptone broth with test organism.
2. Incubate for 48 hours at 35-37^{o}C.
3. Add 4-5 drops of the Kovac's reagent or Ehrlich's reagent.
4. Shake gently and examine for production of a red colour which indicates a positive reaction.

10.6.4 Results

Red colour	Positive Test (Indole produced)
No red colour	Negative Test (No indole produced)

10.6.5 Quality Control

E. coli is used as a positive control while Enterobacter aerogenes serves as a negative control.

10.7 UREASE TEST

This test is used for differentiating between enterobacteriaceae. Proteus strains are urease positive (produce urease enzyme) whereas salmonella, shigella and E. coli are negative (do not produce urease enzyme).

10.7.1 Principle

A culture media containing urea and indicator phenol red is used to culture test organism. If the bacterium is urease producer, the urea will be converted to ammonia and carbon dioxide. The media is turned alkaline after the release of ammonia and can be seen by a change in colour of indicator to red.

10.7.2 Requirements

- Motility indole urea (MIU) medium
- Wire loop
- Indole paper strip
- Test tube

10.7.3 Procedure

1. With the help of a sterile straight wire, inoculate a tube of sterile MIU media with a smooth colony of the test organism.
2. Place an indole paper strip in the upper portion of the MIU tube above the medium. Stopper the tube and incubate at 37^0C overnight.
3. Observe for the formation of urease enzyme by looking for red-pink colour in the medium.

10.7.4 Results

Red-pink colour	Positive
No red-pink colour	Negative

10.7.5 Quality Control

Known positive samples (Proteus vulgaris) and negative samples (E.coli) are used to verify the result.

10.8 LESS COMMON BIOCHEMICAL TESTS

10.8.1 Litmus milk decolourization test (LMDT): This test is done to assist in identification of enterococci and some clostridia. These bacteria have the capacity to metabolize litmus milk.

10.8.2 Lysine decarboxylase test (LDT): This test is used to help in identifying salmonellae and shigellae.

10.8.3 Beta-glucuronidase test : This test is used in identification of Escherichia coli. This bacterium produces the beta-D-glucuronidase, which hydrolyzes beta-D-glucopyranosid-uronic derivatives to aglycons and D-glucuronic acid.

11. CULTURE MEIDA

The bacteria are isolated by growing them on the surface of a culture media. Such a medium is usually composed of a mixture of protein digests (e.g., peptone, tryptone) and inorganic salts which are made harder by adding agar. Standard or general purpose media will aid in the growth of a broad variety of bacteria. The culturing of micro-organisms helps in identification and if indicated, tested for their sensitivity.

11.1 COMMON INGREDIENTS OF A CULTURE MEDIA

11.1.1 Peptone

This is a general term used for the waters soluble products obtained from the hydrolysis of animal or plant proteins. The hydrolysis is due to acids or enzymes. The products are free amino acids, peptides and proteases. Peptone provides nitrogen for growing micro-organisms. Plant proteins also provide carbohydrates. Peptone powder should be light in colour, dry and have a neutral PH.

11.1.2 Meat Extracts

Beef extract such as LAB LEMCO provides a further supply of amino acids and essential growth vitamins and minerals including phosphates and sulfates.

11.1.3 Yeast Extracts

This is contained in many culture media as a bacterial growth stimulant, e.g. in XLD, MNYC and TCBS medium.

11.1.4 Mineral Salts

For cell growth, sulfates are required as sources of sulfur and phosphates as sources of phosphorus. Mg, K, Fe, Ca and other elements are also required for bacterial enzyme activity.

11.1.5 Carbohydrates

Simple or complex sugars are added to many culture media to provide bacteria with sources of carbon and energy. These are also added to media for identification of bacteria, e.g. lactose is added to MacConkey agar to differentiate enterobacteriaceae.

11.1.6 Agar

This is an inert polysaccharide extract obtained from a variety of red purple seaweeds. For use in culture media, agar must be classified and free from pigments and substances toxic to bacteria. Agar is used to solidify culture media because of its high gelling strength and its setting temperature of 32-39^{o}C. Besides being used to solidify culture media, agar also provides micro-organisms with calcium and other organic ions.

11.1.7 Water

This is essential for the growth of all micro-organisms. It must be free from any chemicals which inhibit bacterial growth. De-ionized or distilled water must be used in the preparation of culture media if the local water supply has a high mineral content.

11.2 TYPES OF CULTURE MEDIA

The main types of culture media are basic, enriched, transport, selective, differential, identification and auxanographic media:

11.2.1 Basic Media

The basic media are also called simple media and include nutrient agar which aid in the growth of microbes that do not have special nutritional prerequisites. Basic media are helpful in:

- Production of enriched media;
- Maintaining stock cultures of control strains of bacteria; and
- Sub-culturing pathogens from differential or selective media before performing tests of identification (biochemical and serological).

11.2.2 Enriched Media

An enriched medium enhances growth-promotion in certain microorganisms. These organisms require ingredients like blood, glucose, vitamins and special extracts in order to grow. Enriched media contains these ingredients and assist in helping organisms grow. An enriched medium increases the number of pathogen by containing all the necessary ingredients to promote its growth. Such a medium is often used for specimens collected from sites which are normally sterile to ensure the rapid growth of a pathogen which may be present only in small numbers. Enriched media may also be converted to selective or differential.

11.2.3 Selective Media

These media permit the growth of some bacterial types and inhibit the growth of others. The ability of selectivity is achieved in various ways, e.g. microbes which use a given sugar are easily screened by making that sugar the only carbon source in the culture media. The ability of inhibition is achieved by adding some kind of dyes, antibiotics, salts or specific inhibitors which have an effect on the metabolism or enzyme systems of the microbes, e.g. media containing potassium tellurite, sodium azide or thallium acetate inhibit the growth of Gram-negative bacteria. In case of XLD agar, it selects for Salmonella and Shigella by having bile salts that stop the growth of many faecal commensals. Examples of antimicrobial selective media include Modified New York city (MNYC) medium for isolating N. gonorrhoeae from urogenital specimens and Butzler medium for isolating Campylobacter species from faeces.

11.2.4 Differential (Indicator) Media

These media are used to differentiate microbes which are closely related. Depending on the presence of some special dyes or chemicals in the media, the microbes will produce specific changes or growth patterns that are helpful in the identification or differentiation., e.g. TCBS agar has the indicator bromothymol blue to differentiate sucrose fermenting from non-sucrose fermenting vibrio species. Blood agar can also be described as a differential medium when it differentiates haemolytic from non-haemolytic bacteria. Many culture media are both differential and selective such as XLD agar, TCBS agar, MacConkey agar and DCA.

11.2.5 Transport Media

These are frequently semisolid media containing ingredients to stop the overgrowth of commensals and guarantee the survival of aerobic and anaerobic pathogens when specimens are not cultivable soon after collection. They are used for transporting microbiological specimens from collection sites to microbiology laboratory. Such media lack carbon, nitrogen, and organic growth factors so as to prevent microbial multiplication. They contain only buffers and salts and temporarily store specimens. Examples of transport media include Cary-blair medium for preserving enteric pathogens and Amies transport medium for ensuring the viability of gonococci.

11.2.6 Identification Media

These include media to which substrates or chemicals are added to help identify bacteria isolated on primary cultures. Examples include peptone water sugars, urea broth, and kligler iron agar. Organisms are mainly identified by a change in colour of the medium or the production of gas. It is important that organisms must be first isolated in pure culture before being inoculated on an identification media.

11.2.7 Auxanographic Media

These are defined media lacking in certain nutritional factors. The organism is plated onto the medium and various nutritional factors are spotted onto the medium. Growth in and around these areas indicates the need of the organisms for that particular factor. Identification certain organism can be carried out by this method.

11.3 SOLID, SEMI-SOLID AND FLUID CULTURE MEDIA

11.3.1 Solid Media

Media are solidified by incorporating a gelling agent such as agar or gelatin. Agar is used because of its high gelling strength, its setting temperature of 32-39^{o}C and melting temperature of 90-95^{0}C. Solid media are mainly used in Petri dishes as plate cultures. When grown on solid media, micro-organisms multiply to form visible colonies. Colonial appearances and any changes in the surrounding media help to identify bacteria and differentiate commensals from pathogens. Some cultures also have a distinctive smell, e.g. those of Proteus and P. aeruginosa.

11.3.2 Semi-Solid Media

This form of medium is prepared by adding a small amount of agar (0.4-0.5%w/v) to fluid medium. Semisolid media are used mainly as transport media and for motility testing. Examples are Cary-Blair and Amies medium.

11.3.3 Fluid Media

These are most commonly used as enrichment where organisms are likely to be few, e.g. blood culture. These may also be used for biochemical testing. The growth and multiplication of bacteria in a fluid medium is usually described in four stages, or phases, as follows:

Lag Phase: In this phase, strong metabolic activity occurs but cells do not divide.

Logarithmic Phase: This phase is characterized by rapid cell division. Antibiotics act best at this phase because cells are making peptidoglycan, i.e. they are dividing.

Stationary Phase: The number of cells produced equals the number of cells die due to nutrient depletion or toxic products.

Death Phase: In this phase, there is a decline in the number of viable bacteria.

11.4 BASIC SET OF CULTURE MEDIA USED IN ROUTINE LABORATORY

11.4.1 Blood Agar

This non-selective agar is used for detection of haemolytic activity of the microorganisms. They contain a peptone mixture particularly adapted to the culture of fastidious microorganisms. Blood agar can be used to subculture

bacterial strains in order to obtain pure cultures. There are three types of haemolysis which can be observed:

- Alpha haemolysis is a green halo around colony, e.g. Streptococcus pneumoniae.
- Beta haemolysis is a clear, colourless zone which appears around the colonies, indicating that red blood cells have undergone absolute lysis, e.g. Staphylococcus aureus.
- Gamma haemolysis is normal-looking colony, e.g. Micrococcus luteus.

11.4.2 MacConkey Agar

This culture medium demonstrates the capability of a gram negative bacterium to metabolize Lactose. MacConkey agar is both a selective and differential medium commonly employed in culture testing. It is prepared by mixing 5% sheep's red cells to a complex medium containing peptones just before the plates are poured. The agar has a dye (crystal violet) along with bile salts. Both of these ingredients are responsible for inhibiting the growth of most gram positive bacteria. The agar also contains a sugar, lactose and an indicator, allowing for differentiating. E. coli and Enterobacter aerogenes form lactose fermenting colonies (pink to red colonies) while Shigella, Salmonella and Pseudomonas form non-lactose fermenting colonies (transparent to off white in colour).

11.4.3 Mueller Hinton Agar

This non-selective and non- differential culture medium is used for antibiotic susceptibility testing. It will support the growth of all microbes. The medium contains starch which absorbs the toxins which bacteria release during growth. Therefore, no cross reaction with antibiotics.

11.4.4 CLED Agar

The CLED (cysteine lactose electrolyte deficient) agar is a non-inhibitory culture medium employed for the isolation of urinary bacteria. Yellow coloured colonies are shown by lactose fermenters while blue colour in case of non-lactose fermenters.

12. ANTIMICROBIAL SUSCEPTIBILITY TESTING

In order to carry out antibacterial therapy on a rational basis, physicians rely greatly on information from the clinical microbiology laboratory for treatment of their seriously patients. They needs to have information not only about the precise nature of infection and the identity of the infecting organism but also accurate and reliable guidance regarding specific antibiotics which can be effectively used against that particular organism. Therefore, one of the most important functions of a clinical microbiology laboratory is to provide such information to the clinicians in order to help them in selecting appropriate antimicrobial therapy in a particular situation. This task is accomplished by performing in vitro antimicrobial susceptibility tests in the laboratory against the particular organism that has been identified as the etiological infecting agent. Muller-Hinton agar is recommended for bacterial susceptibility. For Streptococcus pneumoniae strains de-fibrinated sheep blood agar is used, and for Haemophilus species, chocolate agar is used. Antimicrobial susceptibility is performed mainly by two methods:

12.1 MINIMUM INHIBITORY CONCENTRATION (MIC) TECHNIQUE

It is the minimum concentration of an antimicrobial agent (expressed in micrograms or units per ml) that will inhibit the growth of an organism in vitro. MIC results are quantitative rather than qualitative and provide a susceptibility result that is more closely related to *in vivo*. In the MIC method, dilutions of an antimicrobial are added to a broth or agar medium to which standardized inoculums of the test organism is then added. After overnight incubation, the inoculated medium is examined for growth. The lowest concentration of the antimicrobial that prevents visible growth is reported as MIC.

12.2 DISC DIFFUSION TECHNIQUE

Agar disc diffusion method is commonly used throughout the world because it is convenient and inexpensive to perform. Filter paper discs of standard diameter are impregnated with appropriate concentrations of an antimicrobial agent. Antimicrobial discs are applied to the inoculated plate and incubated. The antimicrobial agent diffuses from the disc into the medium. If the organism is susceptible to the drug, growth around the disc will be inhibited to give a zone of clearance.

12.3 MODIFIED KIRBY-BAUER TECHNIQUE

For the performance of the procedure, select growth from a purity plate or an isolated colony from a primary plate and make a suspension in sterile normal saline or distilled water. The suspension should match the turbidity standard # 0.5. If turbidity standard is not available, suspension can be standardized in a 75x10 mm sterile tube. Normal prints (as used in books) should just be visible through the suspension. Dip a sterile swab in this suspension, mop on the side of the tube to drain any excess suspension and inoculate a fresh dried plate of Mueller-Hinton agar evenly, rotating the swab while inoculating. Apply appropriate set of antibiotic discs according to the organism isolated (gram positive or gram negative as the case may be). Incubate overnight and note down the zone diameters next morning and report as Susceptible (S), Intermediate (I), or Resistant (R) according to the standards laid down for each antibiotic.

13. ISOLATION OF PATHOGENS FROM CLINICAL SPECIMENS

13.1 ELEMENTS OF MICROBIOLOGICAL DIAGNOSIS

Collection and Transport of Appropriate Specimens: This is the first and the most important step in microbiological investigations because the results of laboratory examination can only be as good as the specimen we receive.

Microscopy: Direct microscopic examination of a stained smear for microorganisms or an unstained preparation for motility or pus cells, etc. is a useful initial step.

Culture and Sensitivity: isolation and identification of pathogenic organisms on a suitable culture media to find out the nature of infection and to perform antimicrobial susceptibility tests, is the most crucial step in microbiological diagnosis.

Antigen Detection: Some infections can be accurately diagnosed by direct detection of specific antigens of infecting organisms. Examination of CSF for antigens of N. meningitidis, H. influenza and S. pneumoniae is a highly reliable procedure for the diagnosis of bacterial meningitis.

Serological Diagnosis: Detection of specific antibodies in the serum is an established diagnostic procedure for many infectious diseases.

Molecular Diagnosis: Nucleic acid testing by PCR (Polymerase Chain Reaction) has revolutionized the medical diagnosis in many fields including clinical microbiology.

13.2 PATHOGENS OF MAJOR CLINICAL INTEREST (PRIORITY PATHOGENS)

The pathogens of clinical interest can be groups as follows:

Gram positive pyogenic cocci

- Staphylococcus aureus
- Streptococcus pyogenes
- Streptococcus pneumoniae
- Enterococcus

Gram negative pyogenic cocci

- Neisseria meningitidis
- Neisseria gonorrhoea

Gram negative enteric rods

- Salmonella
- Shigella
- E. coli
- Enterobacter
- Proteus
- Klebsiella

Gram negative non-enteric rods (cocco-bacilli)

- Pseudomonas
- Acinetobacter
- Haemophilus

Gram positive bacilli

- Corynebacterium diphtheriae
- Bacillus anthracis

13.3 PROCESSING OF CLINICAL SPECIMENS: SUMMARY OF INITIAL PROCEDURES

Specimen	Pathogens	Microscopy	Media	Incubation
Blood	Salmonella Enteric GN rods Pyogenic cocci Haemophilus Pseudomonas	Not required	Brain heart infusion (BHI) or TS broth	Incubate 7-10 days Subculture to chocolate agar and MacConkey agar after 1-2 days or when broth shows turbidity
Urine	Gram negative rods Staph saprophyticus Enterococcus Pseudomonas	Examine deposit for pus cells	Blood agar plus MacConkey or CLED	Use calibrated loop or Bacteriuristrip Report colony count
Pus/Wound Swab	Staph aureus Strep pyogenes Pseudomonas Gram negative rods Enterococcus	Gram stain	Blood agar MacConkey	Aerobic
Throat Swab	Beta haemolytic Streptococcus Corynebacterium	Albert stain	Blood agar Löffler's medium	Candle jar

	diphtheriae			
HVS	Candida Trichomonas	Wet preparation Gram stain Nugent score	Chocolate agar	Candle jar
CSF	N. meningitidis Strep pneumoniae H. influenzae Strep Group B E.coli Enterococcus	Cell count Gram stain	Blood/Chocolate agar MacConkey agar	Incubate chocolate agar in candle jar
Stool	Shigella Salmonella		Selenite or tetra broth. MacConkey SS or XLD	Identify non-lactose fermenting colonies
	Vibrio cholerae	Microscopy for motility	APW TCBS agar MacConkey	See motility after 6-8 hours in APW. Subculture to MacConkey/TCBS

13.4 IDENTIFICATION OF CLINICAL ISOLATES

Complete identification of all clinical isolates is the cherished goal of all microbiology laboratories. However, in the majority of routine hospital laboratories in our country, it is neither possible and cost effective nor absolute necessary unless required for epidemiological purposes. The clinicians will be more interested in knowing the exact antimicrobial susceptibility profile of the infecting organisms rather than the precise identification.

Having said that, it should be possible for most routine laboratories to identify majority of clinically important isolates up to the genus level, using the basic tests of characteristics described in this manual. Commercially available systems like AP1 are costly but extremely selective and if a laboratory can afford them, they may be reserved exclusively for identification of crucial isolates like those from blood and CSF or for NLF urease negative isolates from stool specimen suspected to be Salmonella or Shigella.

14. BLOOD CULTURE

This is a microbiological culture of human blood. The test is used to identify infections which are present in the bloodstream (such as bacteraemia, septicaemia). When signs and symptoms of a systemic infection are present, the blood culture test assists in identifying the microbial cause of the infection. The test can also detect the type of organism causing infection.

Septicaemia is the term used to describe bacterial toxin in the blood stream usually released by actively multiplying bacteria (exotoxin) or by the immunological reaction of cytokines and chemokines released by lymphocytes and other blood cells (endotoxin). On the other hand, bacteraemia is the existence of bacteria in bloodstream which can occur spontaneously (during certain tissue infections, with the application of IV catheters, after dental treatment, wound-care, or other procedures) and metastatically.

14.1 Requirements

- Tryptic Soy Broth or Brain heart infusion broth containing 0.25% SPS (Sodium polyanethol sulphonate) - 20 ml for children, 40 ml for adults
- Media for subculture: Blood agar, MacConkey agar, Chocolate agar
- Sensitivity test medium: Muller Hinton agar, Blood agar
- API 20E or API 10S

14.2 Specimen Collection

About 10 ml of blood is collected via venepuncture method and put into two or more blood collection bottles containing specific media for aerobic and anaerobic micro-organisms. A frequently used media for anaerobes is thioglycollate broth. The following precautionary measures should be taken while collecting sample for blood culture:

- The blood culture bottle should be pre-warmed at room temperature.
- The venepuncture site should be decontaminated for 1 – 2 minutes with 70% alcohol. Similarly the bottle cap rubber stopper should be decontaminated with 70% alcohol thoroughly and allow to air dry.
- Anticoagulated blood should be avoided.
- Before collecting blood for culture antibiotic therapy should be withdrawn.

14.3 Culturing

The inoculated blood culture bottle should be incubated at 37^0C for 5-7 days before reporting as negative. The bottle should be observed daily for turbidity. If the upper layer of the bottle becomes turbid, sample should be sub-cultured on Blood agar, MacConkey agar, and Chocolate agar. After 24 hours, the subculture plate should be observed for growth. If there are visible colonies on plate, the specimen plate should be dealt according to the following identification scheme:

14.3.1 Staining

A slide should be made of bacterial colony and air dried. The slide should be stained with Gram stain and observed under microscope.

14.3.2 Biochemical Testing (if colony is Gram Positive)

If the colony is gram positive, then perform catalase test to differentiate between Staphylococcus (catalase positive) and Streptococcus (catalase negative). If the bacteria colony is confirmed as Staphylococcus species, then perform coagulase test to differentiate between Staphylococcus aureus (coagulase positive) from Staphylococcus epidermidis (coagulase negative).

After species identification, appropriate sensitivity disc should be selected to perform susceptibility testing.

14.3.3 Biochemical Testing (if colony is Gram Negative)

If the colony is gram negative, oxidase test is performed to differentiate between oxidase positive (pseudomonas specie) from oxidase negative (E. coli). Similarly urease test is performed to differentiate between urease positive from urease negative and indole test is used to differentiate between indole positive from indole negative. If there is ambiguity in such identification then API 10 or API 20 is used for the definitive identification. After the identification of species, a panel of antibiotics disc is selected to perform susceptibility testing.

14.4 Commonly Isolated Microorganisms

Gram negative	**Gram positive**
Salmonella typhi	Staphylococcus aureus
Other Salmonellae	Streptococci virdans
Enteric rods	Streptococcus pneumonia
H. influenzae	Streptococcus pyogenes
N. meningitides	Enterococci
Pseudomonas aeruginosa	

14.5 Positive Culture

More than 90% of blood cultures containing true pathogen become positive within the first 48 hours of inoculation. Blood cultures taking more than 72 hours to become positive are more likely to be contaminants. Positivity rate is 5-15% of all blood cultures while 3-5% contaminants among all blood cultures. The contaminants include the coagulase negative Staphylococci from skin during venepuncture.

15. URINE CULTURE

Urine culture is a microbiological culture of urine. It is applied to detect infections that cause urinary tract infections (UTIs) and disseminated infections (Pyuria, Burning urine, low grade fever with increase urination, after following catheterization, after urosurgical procedures, un-explained dysuria). UTIs are usually classified into upper tract infections (involving kidneys) and lower tract infections (involving bladder, urethra, or prostate). Symptoms can be absent or may be present including urinary frequency and urgency, dysuria, lower abdominal pain, and flank pain. The laboratory diagnosis relies on analysis and culture of urine.

15.1 Requirements

- CLED agar is used as a single urine culture medium. Alternately, Blood agar and MacConkey agar may also be used. For sensitivity test, Muller Hinton agar is used.
- Oxidase reagent
- Coagulase Plasma
- API kit/ Biochemical set

15.2 Specimen Collection

For urine culture, mid stream urine is collected. The urethral opening is washed with a mild, non-foaming disinfectant and air dried. Contact of the urinary stream with the mucosa should be minimized by spreading the labia in women. Urine specimen must be cultured within 1 hour of collection. If delay is anticipated, specimen can be refrigerated for up to 24 hours.

15.3 Quantitative Culturing

Calculated loop (1 ul or 10 ul) of specimen (or MAST Uri System) is initially inoculated on CLED agar. The culture plate is incubated at 37^0C for 24 hours initially. The time of incubation is extended up to 48 hours before reporting negative. The scheme of organism identification is similar for nearly all bacteriological specimens with some exceptions.

Colony count using 1 ul loop: > 100 colonies are significant and indicate 10^5 bacteria/ml which is an index of true UTI.

15.3.1 Staining

A slide should be made of bacterial colony and air dried. The slide should be stained with Gram stain and observed under microscope.

15.3.2 Biochemical Testing (if colony is Gram Positive)

If the colony is gram positive then perform catalase test to differentiate between Staphylococcus (catalase positive) and Streptococcus (catalase negative). If the bacteria colony is confirmed as Staphylococcus species, then perform coagulase test to differentiate between Staphylococcus aureus (coagulase positive) from Staphylococcus epidermidis (coagulase negative).

After species identification, appropriate sensitivity disc should be selected to perform susceptibility testing.

15.3.3 Biochemical Testing (if colony is Gram Negative)

If the colony is gram negative then oxidase test is perform to differentiate between oxidase positive (pseudomonas specie) from oxidase negative similarly urease test is perform to differentiate between urease positive from urease negative and indole test is used to differentiate between indole positive from indole negative. If there is ambiguity in such identification the API 10 or API 20 is used for the definitive identification. After the identification of species a panel of antibiotics disc is selected to perform susceptibility testing.

15. 4 Commonly Isolated Microorganisms

Gram negative	**Gram positive**
E. coli	Enterococcus
Klebsiella species	Staphylococcus saprophyticus
Proteus species	Staphylococcus aureus
Enterobacter	Beta haemolytic streptococcus
Acinetobacter	
Citrobacter	
Serratia	
Pseudomonas	

16. HIGH VAGINAL SWAB (HVS) CULTURE

HVS culture is a microbiological culture of vaginal swab. It is applied to detect infections that cause vaginal infections. Women with vaginal infection (vaginitis) commonly present with excessive, abnormally discoloured discharge, malodour, irritation, itch, swelling and discomfort. Irregular or unexpected bleeding and abdominal pain may indicate the presence of more serious disease. The clinical picture, through a precise history and genital examination, will usually reveal the cause, but diagnosis can be confirmed by tests such as high vaginal swab culture. The causative organisms can be divided into sexually transmitted and non-sexually transmitted. Over-proliferation of commensal organisms, most commonly candidal yeasts such as Candida albicans, and commensal bacteria in bacterial vaginosis (BV), can cause vaginal symptoms in otherwise healthy women of any age.

16.1 Requirements

- High Vaginal Swab
- Chocolate agar
- CO_2 jar
- MacConkey agar
- Blood agar

16.2 Specimen Collection

As the name HVS indicates (high vaginal swab), a swab containing specimen from vagina. The specimen is usually collected by a lady doctor or gynae associate nurse.

16.3 Culturing

Swab containing specimen is inoculated on Chocolate agar. The culture plate is incubated at 37^0C for 24 hours initially in CO_2 jar or CO_2 incubator. The time of incubation is extended up to 72 hours before reporting negative. HVS is usually cultured for candidiasis but disseminated infections can also be ruled out using blood and MacConkey agar. The scheme of organism identification is similar for nearly all bacteriological specimens with some exceptions.

For the wet preparation, the same swab after inoculation, is used for making a wet preparation slide for microscopy to detect motile Trichomonas.

16.3.1 Staining

A slide should be made of bacterial colony and air dried. The slide should be stained with Gram stain and observed under microscope.

16.3.2 Biochemical Testing (if colony is Gram Positive)

The Candida is gram positive and identified by specific morphological characters under the microscope.

If the colony is gram positive other than Candida, then perform catalase test to differentiate between Staphylococcus (catalase positive) and Streptococcus (catalase negative). If the bacteria colony is confirmed as Staphylococcus species, then perform coagulase test to differentiate between Staphylococcus aureus (coagulase positive) from Staphylococcus epidermidis (coagulase negative).

After species identification, appropriate sensitivity disc should be selected to perform susceptibility testing.

16.3.3 Biochemical Testing (if colony is Gram Positive)

If the colony is gram negative, oxidase test is performed to differentiate between oxidase positive (pseudomonas specie) from oxidase negative (E. coli). Similarly urease test is performed to differentiate between urease positive from urease negative and indole test is used to differentiate between indole positive from indole negative. If there is ambiguity in such identification then API 10 or API 20 is used for the definitive identification. After the identification of species, a panel of antibiotics disc is selected to perform susceptibility testing.

16.4 Commonly Isolated Microorganisms

- Candida albicans
- Trichomonas vaginalis
- E. coli
- Pseudomonas aeruginosa
- K. Pneumoniae
- Staph. aureus

The Candida albicans and Trichomonas vaginalis are considered to be the only true pathogens causing vaginitis and vaginal discharge.

17. THROAT SWAB CULTURE

The main aim of performing a throat culture is the identification of specific microbes causing a sore throat or throat infection. The test is also helpful in the identification of group A, beta-haemolytic streptococci. The test also aids in the identification of carriers (infected individuals who do not show symptoms). It is important to note that 70% of all sore throats are of viral origin.

17.1 Requirements

- Culture medium: Blood agar prepared in an agar base without glucose. Blood from any species including human (donor blood) can be used. Sheep blood is preferred because it inhibits growth of haemolytic Haemophilus and also that of a haemolytic variant of Enterococcus.
- Bacitracin discs – 0.04 units
- Candle jar
- Gram stain set

17.2 Specimen Collection

The tongue depressor is used to expose the throat and sterile swab is rubbed firmly on the posterior pharynx and on both the tonsillar areas avoiding touching the cheeks, tongue or lips. The swab should be cultured as soon as possible. In case of delay insert the swab in a tube containing silica gel (common dessicant). In silica gel, S. pyogenes remain viable for up to 3 days.

17.3 Culturing

Throat swab is inoculated on blood agar and the wire loop is streaked in a fashion that primary, secondary and tertiary streaks are well separated and isolated single colonies of bacteria are easily obtainable. The bacitracin disc (0.04 units) and a co-trimoxazol sensitivity disc are placed on the secondary streak (or at the junction of primary and secondary streak). The sensitivity plate is placed in a CO_2 jar. The closed jar containing culture plate is incubated at 37^0C for initially 24 hours and extended up to 48 hours before reporting as negative.

17.4 Identification

After 18-24 hours incubation, blood agar plate should be examined in transmitted light to note the presence or absence of beta haemolytic colonies. Normally, throat swab always yields a heavy mixed growth of commensal organisms.

Beta haemolytic colonies are seen which are of Streptococcal species and are small, 0.5-1 mm in diameter, circular slightly opaque and appear as dew drops or minute beads of moisture on a moist agar surface. Streptococcus pyogenes is sensitive to 0.04 unit penicillin (any zone of clearance is significant) but resistant to co-trimoxazol but majority of other throat bacteria are sensitive.
99% Group A Streptococcal strains are selectively inhibited by bacitracin. If the organism is resistant to bacitracin, it indicates beta-haemolytic Streptococcal species and other Group A Streptococci.

17.5 Commonly Isolated Microorganisms

Gram positive

- Streptococcus pyogenes (occasionally Streptococcus Group B, C and G)
- Corynebacterium diphtheria

Gram negative

Gram negative bacteria from throat swab are very rarely isolated.

18. PUS/WOUND CULTURE

This is a microbiological culture of skin lesion or skin aberrations. The test is used to identify infections which are present in the wound/pus or purulent cut on skin. The infection is usually localized, up till now no case of disseminated infection has been reported. Wound culture or pus culture is advised usually in delayed wound healing or wound of diabetic patients to prevent from being infected with bacterial or fungal infection.

18.1 Requirements

- Blood Agar, Chocolate Agar, MacConkey Agar
- Sensitivity test medium: Muller Hinton agar, Blood agar
- API 20E or API 10S

18.2 Specimen Collection

For specimen collection, the wound is opened with the help of twizzer and swab is applied on the wound. After collection, the specimen is transported as soon as possible for examination.

18.3 Culturing

The swab is inoculated on Blood agar, MacConkey agar, and Chocolate agar and incubated at 37^0C for 3 days before reporting as negative. If there is growth on plate, the specimen plate should be dealt according to the following identification scheme:

18.3.1 Staining

A slide should be made of bacterial colony and air dried. The slide should be stained with Gram stain and observed under microscope.

18.3.2 Biochemical testing (if colony is Gram Positive)

If the colony is gram positive, then perform catalase test to differentiate between Staphylococcus (catalase positive) and Streptococcus (catalase negative). If the bacteria colony is confirmed as Staphylococcus species, then perform coagulase test to differentiate between Staphylococcus aureus (coagulase positive) from Staphylococcus epidermidis (coagulase negative).

After species identification, appropriate sensitivity disc should be selected to perform susceptibility testing.

18.3.3 Biochemical Testing (if colony is Gram Negative)

If the colony is gram negative, oxidase test is performed to differentiate between oxidase positive (pseudomonas specie) from oxidase negative (E. coli). Similarly urease test is performed to differentiate between urease positive from urease negative and indole test is used to differentiate between indole positive from indole negative. If there is ambiguity in such identification, then API 10 or API 20 is used for the definitive identification. After the identification of species, a panel of antibiotics disc is selected to perform susceptibility testing.

18.4 Commonly Isolated Microorganisms

Gram negative	**Gram positive**
Enterobacteriaceae	Staphylococcus aureus
Pseudomonas aeruginosa	Staphylococci pyogenes
	Candida albicans

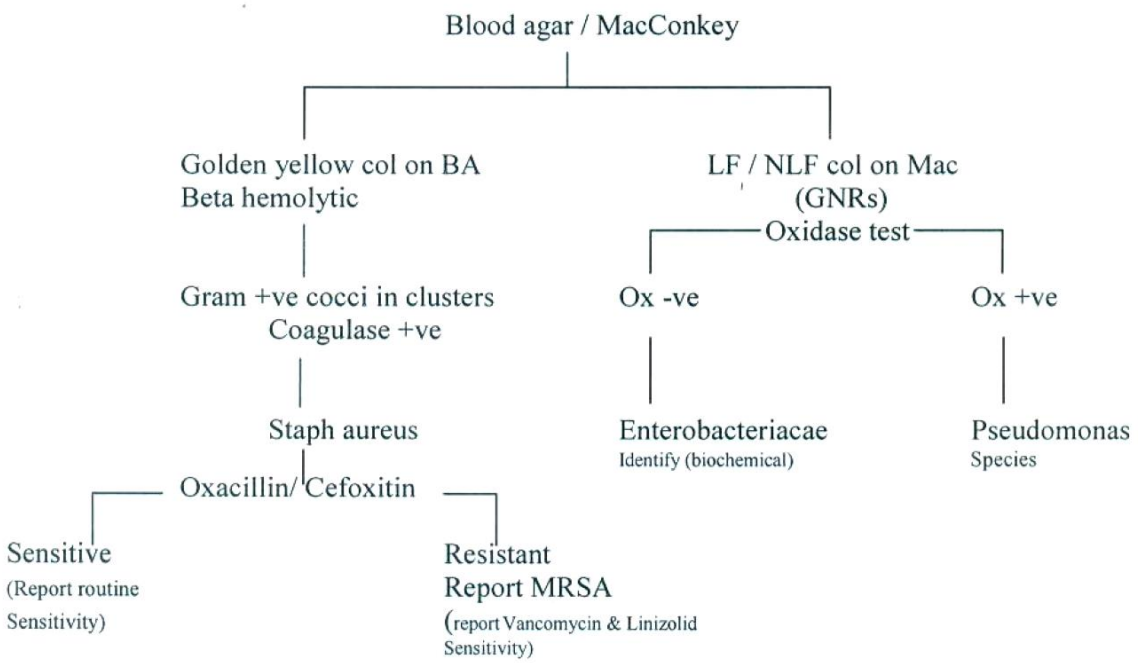


Fig 18.1 Flow diagram of pus/wound culture processing

19. STOOL CULTURE

Stool culture is requested when there is an infection of lower digestive tract. Stool sample is collected in clean sterile container and a sample is inoculated as soon as possible because if there is a delay, there would be overgrowth of commensals. Therefore, if there is delay in specimen inoculation, the samples must be refrigerated.

19.1 Requirements

- SSA agar/DCA/ XLD agar or MacConkey agar,
- TSI or KIA
- Peptone water for Indole

19.2 Specimen Collection

Stool sample should be collected in a sterile tight fitting leak proof container and processed as soon as possible upon receipt in the laboratory but no longer than 2 hours after collection.

19.3 Culturing

The sample is inoculated on SSA agar, MacConkey agar, and TSI agar and incubated at 37^0C for 3 days before reporting as negative. If there is growth on plate, the specimen plate should be dealt according to the following identification scheme:

Examine the plates for non-lactose fermenting (NLF) colonies. On MacConkey, SS agar or DCA, NLF appears as colourless pale colonies while LF colonies are pink or red. On XLD, the LF colonies are yellow and NLF colonies are red. All enteric pathogens like Salmonella, Shigella and Y. enterocolitica produce NLF colonies. Salmonella is motile, produces gas and H_2S in TSI. Shigella is non-motile, does not produce gas or H2S in TSI. Further identification will depend upon the diagnostic facilities available. API is one of the best ID systems available for the identification of enteric pathogens.

Basic Fuchsine satin is performed on slide prepared from fresh stool sample for the identification of Campylobacter Jejuni which is a curved rod usually present in children stool.

19.4 Commonly Isolated Organisms

- Shigella species
- Salmonella species

A summary of processing of stool culture can be seen from the flow diagram below:

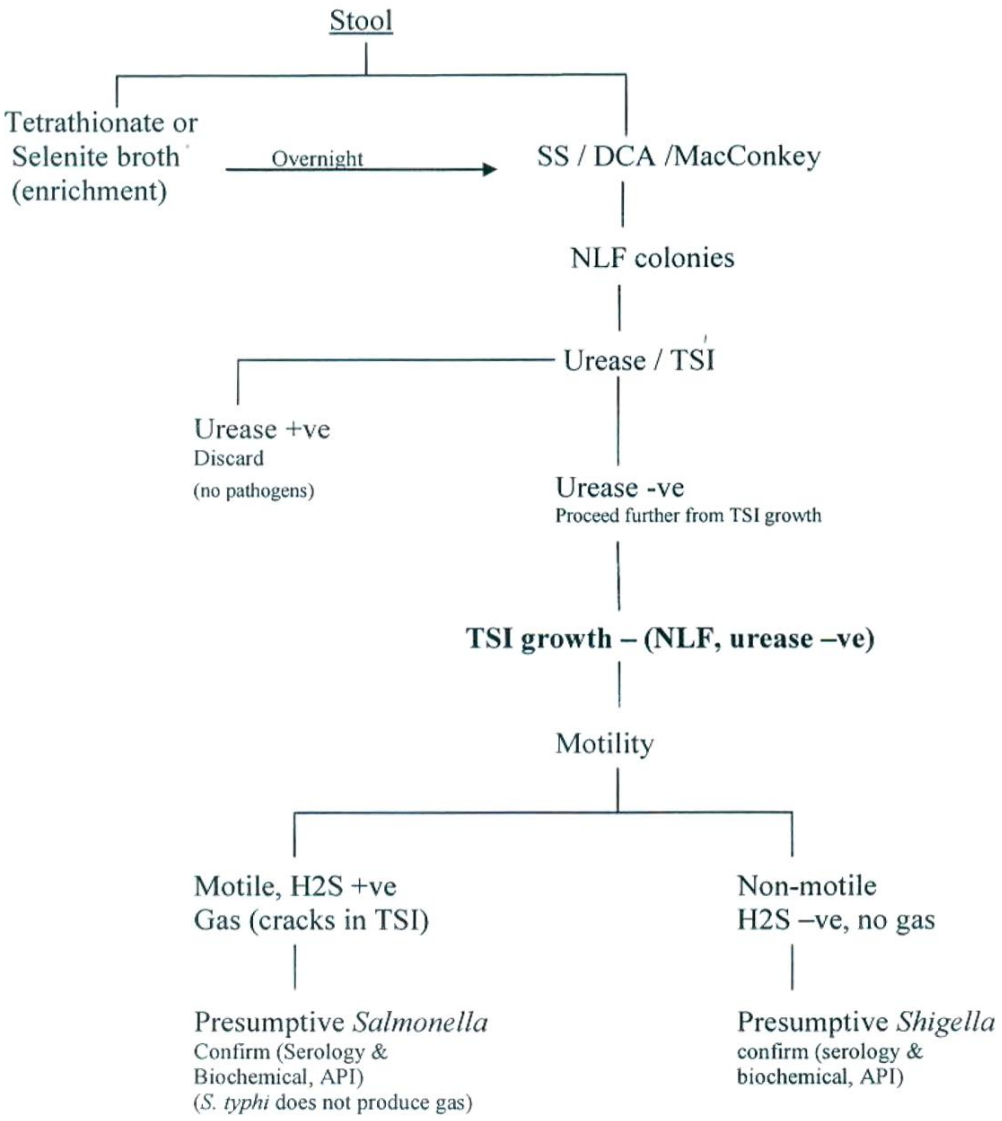


Fig 19.1 Flow diagram of stool culture processing

20. EXAMINATION OF BODY FLUIDS

20.1 CSF ANALYSIS

The cerebrospinal fluid (CSF) is a secretion produced by the choroid plexuses (tuft of capillaries lined by a single layer of cells). It is clear alkaline fluid resembling plasma. It is secreted continuously at a rate of 0.5 ml/minute, i.e.720 ml/day. The protein content is low due to lack of fibrinogen. Chloride is 25% higher than plasma chloride level and glucose is 2/3 rd of the blood sugar. CSF performs various vital functions in the body such as protecting brain and spinal cord, keeping them moist, maintaining uniform pressure around brain and spinal cord and acting as an exchange medium. A broad range of disorders can produce changes in CSF composition, e.g. infections, vascular disorders, trauma and degenerative disorders. The diagnosis of acute meningitis is the main indication for CSF analysis.

20.1.1 COLLECTION OF CSF SPECIMEN

A CSF specimen is taken from a point below the end of the spinal cord, i.e. below the level of the 2nd lumbar vertebra by introducing a lumber puncture needle between 3r^{d} and 4th lumbar vertebrae (by Lumbar Puncture Technique). About 2-4 mil specimen is collected in 3 screw capped bottles that are serially numbered and must be sent to the laboratory immediately. The examination should ideally be done in 1-2 hours. CSF in the 1st bottle is usually contaminated with blood and shall be kept aside. Fluid from 2"^{d} bottle is used for routine biochemical tests while fluid from 3rd bottle is used for bacterial culture. CSF should never be refrigerated especially the portion submitted for bacterial culture. N. meningitidis and S. pneumoniae are killed on exposure to cold.

20.1.2 PHYSICAL EXAMINATION

Normally CSF is crystal clear and colourless and gives no coagulum on standing if kept sterile. When pathogenic conditions are present, abnormalities in appearance may arise in colour, turbidity and the appearance of a coagulum.

20.1.2.1 Colour

The CSF is normally colourless. The presence of blood is the main cause of abnormal colour. Some may be introduced as a result of trauma while obtaining the fluid or in subarachnoid hemorrhage. In later type, the blood is more

homogenously mixed with the fluid than is introduced during collection. In addition, haemolysis frequently occurs and the haemoglobin is converted into bilirubin. Therefore, 24 hours after the haemorrhage, the supernatant fluid obtained on centrifugation becomes yellow coloured which is called Xanthochromia. There are, however, other conditions of xanthochromia.

20.1.2.2 Coagulum

Normal CSF does not clot due to absence of fibrinogen. Fibrinogen is present when blood brain barrier is disturbed and there is substantial increase in protein. In tuberculous meningitis, there is delicate fine clot (cobweb coagulum or pellicle formation) and in purulent meningitis, there is a large clot.

20.1.3 MICROSCOPIC EXAMINATION

20.1.3.1 Cell Count

If CSF is clear, the cells are counted by charging a Neubauer counting chamber with well mixed undiluted fluid. Cells in all the nine WBCs squares are counted. The number of cells counted is approximately the number of cells per cmm of CSF. If the count is expected to be higher, then CSF has to be diluted for cell counting. Diluting fluid is prepared by dissolving 200 mg crystal violet in 100 ml of 10% acetic acid. Method for counting and calculation is same as for counting of WBCs in peripheral blood.

In normal CSF, the cell count is 0 – 5/cmm and these are lymphocytes. The neutrophils and red blood cells are absent normally. In diseased conditions, the number may increase and other normal blood cells may be present, sometimes accompanied by bacterial cells. It has been seen that in acute meningitis there is increase in leucocytes; in bacterial meningitis, cell response is mainly of polymorphs; in viral meningitis and in tuberculous meningitis, the response is mainly lymphocytic; in acute encephalitis, there is increase either in lymphocytes or in mononuclears; malignant cells and sometimes plasma cells may come in various neoplastic and inflammatory conditions including delayed hypersensitivity; and macrophages can be seen in traumatic conditions or mycotic meningitis. Turbidity is observed when marked increase in polymorphs is present.

20.1.3.2 Bacteriological Smears

1. If CSF is turbid, smear are made directly otherwise it is centrifuged, pour off supernatant and re-suspend the sediment in a few drops of CSF left.
2. Prepare at least three smears on glass slides and dry in air.
3. Stain one smear with Leishman's stain for type of WBC (differential counting), second with Gram stain for presence and type of bacteria and the third with acid fast stain for AFB. Acid fast stain should also be done if a pellicle forms on standing.
4. Special preparations can be made if required, e.g. India ink preparation for Cryptococcosis or direct wet preparation for trypanosomes and Neglaria species. Immunofluorescent stains can be used for Haemophilus influenzae and some other organisms.

20.1.3.3 Cultures

Cloudy fluid should be streaked on Chocolate, MacConkey, Blood and Sabouraud's agar and inoculated into blood broth and thioglycollate medium. All media are incubated at 37^0C, some in candle jars for CO_2 atmosphere. Sediment of centrifuged fluid should be cultured on special media for tubercle bacilli and fungi.

20.1.4 CHEMICAL ANALYSIS

Chemical changes in the spinal fluid especially globulins and glucose indicate the nature of meningitis. So, estimation involves determination of total proteins (normal range 15-40 mg/dl), a qualitative test for globulins (usually < 3 mg/dl), glucose (normal range 50-80 mg/dl) and less often measurement of chloride (normal range 120 - 130 mmol/L) and enzymes, e.g. LDH (10% of serum level).

20.1.5 IMMUNOLOGICAL DIAGNOSIS (DIRECT ANTIGEN DETECTION)

Latex agglutination kits are commercially available for direct detection of specific antigens of N. meningitides, S. pneumoniae and H. influenzae (some with additional E. coli detection). These are highly reliable for rapid detection of etiological organisms but are costly and not widely available.

	Gross	Proteins mg/dl	Glucose mg/dl	Cells /cmm
Normal	Clear	15-40	50-80	0-5 Lymphocytes
Bacterial	Cloudy	60-1000	0-45	1000-50000 Mostly neutrophils
Aseptic (Viral)	Clear	Normal or increased	Normal	100-1000 Mostly lymphocytes
TB	Clear/Clot	Moderate rise (45-300)	Normal or decreased	10-1000

Table 20.1 Summary of CSF Findings in Acute Meningitis

20.2 PLEURAL FLUID ANALYSIS

Disseminated lungs and pleural infection is diagnosed by pleural fluid analysis. Normally pleural fluid is required to decrease the friction between pleura and lungs but when there is an accumulation of pleural fluid, there is no space for the expansion of lungs resulting in clinical symptoms. In the microbiology laboratory, four types of procedures are employed to the pleural fluid as follows:

20.2.1 Physical Examination

This includes colour, pH, presence or absence of coagulum, clot, and turbidity. Normally the fluid will be:

- Colour Pale yellow
- Turbidity Clear
- pH 7.60-7.64

20.2.2 Chemical Examination

This includes glucose, total protein and total leucocytes count. The normal values of these parameters are:

- Total Protein < 2 g/dL
- Glucose < 100 g/dl

20.2.3 Microscopic Examination

Microscopically, the fluid is examined for total and differential leucocytes count and presence of any microorganisms (Gram and ZN stain). The microorganisms may include gram positive, gram negative and mycobacterium species. TLC is < 1000 (WBCs) per cubic millimeter.

20.2.4 Culture and Sensitivity

For definitive microbial identification, the pleural fluid is cultured on blood and MacConkey agar. In addition, the method of choice for bacterial identification is inoculation in blood culture bottle containing BHI media.

20.3 ASCITIC FLUID ANALYSIS

Ascitic fluid examination is done in Ascites which is a gastroenterological term for an accumulation of fluid in the peritoneal cavity. Ascites develop when the quantity of ascitic fluid increases in the peritoneal cavity. The volume of ascitic fluid may be increased either from accumulation, or draining of capillaries into the cavity or obstruction of lymphatic vessels. According to the patho-physiological classification ascetic fluid may be classified into two types, i.e. Transudate and Exudate.

Transudates are the fluid containing <30g/L protein and it may occur during acute and chronic liver disease, rheumatic heart disease, congestive cardiac failure and ischemic heart disease. Exudates are the fluid containing >30g/L protein and it may occur during visceral and systemic malignancy, obstruction of visceral veins and lymphatic system, inflammation of pancreas leading to obstruction of reticluendothelial system, tuberculosis infection or any other disseminated infection. In the microbiology laboratory, four types of procedures are employed to the pleural fluid as follows:

20.3.1 Physical Examination

This includes colour, pH, presence or absence of coagulum, clot, and turbidity. Normally the fluid will be:

- Colour Clear - Pale yellow
- Turbidity Clear
- pH 7.60-7.64

20.3.2 Chemical Examination

This includes glucose, total protein and total leucocytes count. The normal values of these parameters are:

- Total Protein 0.3 - 4.0 g/dL
- Glucose 7-10 g/dl

20.3.3 Microscopic Examination

Microscopically, the fluid is examined for total and differential leucocytes count and presence of any microorganisms (Gram and ZN stain). The microorganisms may include gram positive, gram negative and mycobacterium species. TLC is

<300 per cubic millimeter. Neutrophil count is increased during disseminated and un-disseminated bacterial infection. Absolute neutrophil count is helpful in the diagnosis of spontaneous bacterial peritonitis (SBP), in which case neutrophil count is >250 cells/mm^3. Red blood cells are the indicative parameter of haemorrhage which may be due to cirrhosis or invasive bacterial infection or toxic peritonitis from drainage of (drug induced) lymphatic leakage.

20.3.4 Culture and Sensitivity

In a suspected case of complicated infectious condition such as spontaneous bacterial peritonitis, fluid is cultured on BHI media soon after the collection of fluid. If there is delay in inoculation of fluid, the sample should be refrigerated to preserve the microorganisms. The fluid may also be cultured on blood and MacConkey agar for isolation of colonies if there is limitation of the blood culture facilities.

20.4 SYNOVIAL FLUID

The clear filtrate secreted by monocytes and mononuclear cells into the joints is called synovial fluid. The most important role of synovial fluid is to decrease friction between the articular cartilage of synovial joints during movement. Analysis of synovial fluid is requested in the diagnosis of arthritis which can be Infectious Arthritis (due to the dissemination of bacteria, viruses and fungus after systemic infection); arthritis due to thrombophilia; Gouty arthritis (increase in serum uric acid and calcium); and Autoimmune Arthritis (such as rheumatoid arthritis, SLE, and osteoarthritis). The fluid is collected for analysis by a technique called arthrocentesis (or joint aspiration). In the microbiology laboratory, four types of procedures are employed to the synovial fluid as follows:

20.4.1 Physical Examination

Normally the fluid will be:

Colour	Straw-coloured or Colourless
Appearance	Clear
Consistency	viscous
pH	7.5

20.4.2 Chemical Examination

Glucose: Glucose present in synovial fluid is slightly lower than glucose present in blood.

Uric acid: Normally the synovial fluid has the same normal values of uric acid as in blood uric acid; however, uric acid level is increased in gout.

Protein: The synovial fluid has a normal protein level but it may be increased by the presence of bacteria.

LDH: The level of LDH enzyme in synovial fluid is increased by the presence of any type of arthritis.

20.4.3 Microscopic Examination

Microscopically, the fluid is examined for differential leucocytes count and presence of any microorganisms. The microscopic examination of synovial includes.

TLC: Normal synovial fluid has only a few number of WBCs present (<200 mm^3). The number of WBCs is increased in arthritis.

DLC: A differential leucocyte count is done to determine the type of infection.
Crystals: In gouty arthritis, uric acid or calcium oxalate crystals may be present. Birefringent crystals can also be seen (under polarized light).
Gram stain: Normally no microorganism present in synovial fluid the presence of any microorganism in synovial fluid indicates the presence of infection.
ZN Stain: Normally AFB are not present, however, if AFB seen in ZN stain, they should be reported.

20.4.4 Culture and Sensitivity

Usually disseminated infection of joints is a very rare clinical condition. However, in case of infectious arthritis, the fluid may be cultured on Chocolate agar to isolate the microorganism.

21. SEMEN ANALYSIS

The analysis of semen is performed to aid in the diagnosis of infertility, hypogonadism and before artificial insemination. Semen is a thick, white fluid discharged during ejaculation. Seminal fluid or plasma consists of 5% spermatozoa suspended in seminal plasma. The secretion of seminal vesicles is approximately 60% which is viscid, neutral, or slightly alkaline. The yellow colouration is due to the pigment flavin. The prostate contributes around 20% acidic milky fluid (pH 6.5) containing proteolytic enzymes, spermine (bacteriostatic) and acid phosphatase enzyme. These proteolytic enzymes are responsible for coagulation and liquefaction of semen. The contribution of epididymis, cells of vas deference, bulbourethral and urethral glands is around 15%. The epididymis also adds carnitine in the semen which helps in sperm motility. The ability to enter the egg is also provided by the epididymis. Special tests are also performed in exceptional circumstances, e.g. post-coital test, fructose test and antibodies test.

21.1 Sample Collection

There are various methods to collect semen for analysis. The most commonly applied method is by masturbation. Less commonly applied procedures are coitus interrupts, condom collections and epididymis extraction. It is essential to have a 3-5 days period of abstinence because it affects both the quantity and motility of sperms in semen. The collection bottle/container should be clean, with wide mouth and without any water and detergent. The transport temperature of the sample is around 37^0C. Examination should be performed in 2-3 hours.

21.2 Physical Examination

21.2.1 Appearance

Highly viscid, white or gray-white coagulum with musty or acrid odour.

21.2.2 Liquefaction

The semen coagulum liquefies after 10-20 minutes and turns into a turbid viscous fluid. Add 0.2% alpha amylase if it is not liquefied.

21.2.3 pH

Normal pH of the semen is 7.2-7.8 but may become slightly acidic in some pathological conditions (congenital aplasia and infections of vas deference and seminal vesicles).

21.2.4 Volume

Normal volume is 1.5 - 5.0 ml. An increased semen volume is often linked with infertility and results in low sperm count. If the volume is less, it may cause difficulty in poor penetration of cervical mucus. Semen volume is not affected with the period of abstinence.

21.2.5 Viscosity

This is tested by placing a drop of semen from a 10 cm long capillary tube containing 0.1 ml semen. The time taken by the drop to form and leave the capillary tube is a measure of its viscosity.

21.3 Microscopic Examination

21.3.1 Sperm Morphology

1. The sperm morphology is assessed by examining a stained smear slide.
2. The smears are prepared similar to blood smear.
3. Fix the smear in 95% ethanol before drying.
4. Stain with Papanicolaou stain. Other staining techniques include Mayer's Haemotoxylin, Giemsa, basic fuchsin and crystal violet.
5. Examine a minimum of 200 spermatozoa using oil immersion objective.
6. Note down the percentage of abnormal sperms (e.g. bifurcated tail, pointed head, curved neck, double head, double tail, abnormal middle piece).
7. It is normal to observe 30% abnormal forms.
8. The presence of red cells, white cells and epithelial cells is also noted. Numerous granules and globules are normally present in the semen.

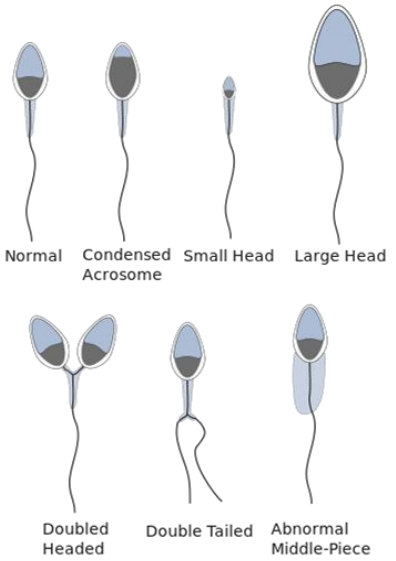


Fig 21.1 Morphology of Spermatozoa

21.3.2 Sperm Counting

1. Dilute the sample in a diluting fluid which consists of sodium bicarbonate, formalin and distilled water. This diluting fluid immobilizes the sperms and assists in counting.
2. The dilution is made with the help of a Thoma white cell pipette (1:20). Dilution can be prepared in a test tube.
3. After dilution, charge the Neubauer counting chamber.
4. Wait for 2 minutes to allow the sperms to settle down.
5. The sperms are counted in large four squares. The count is multiplied with 50 to get the result in cubic millimeters.
6. In case the semen is highly viscous, add a mucolytic agent in equal amount and multiply the final result with 2.
7. Normal sperm count range is 20 - 150 million/ml. (Average 100 million/ml).

21.3.3 Sperm Motility Testing

1. A drop of liquefied semen is kept on a pre-warmed slide and cover slip is placed.
2. Count a minimum of 200 spermatozoa.
3. The whole depth of the fluid should be screened and the non motile sperms settled at the bottom also be included to assess motility.
4. The percentage of sperms showing actual progressive motion is to be recorded.
5. The count should be performed in duplicate and the average is recorded.
6. Normal semen contains about 70% motile sperms.
7. For the calculation of motility score, semen is transferred into a caped tube.
8. Mix by inverting the tube several times and pipette one drop of semen onto a clean glass slide and place cover slip on it.
9. Examine with 40x objective and estimate the percentage of spermatozoa moving at different speeds. This speed can be classified into 4 motility grades as follows:
    - Grade 0: No movement at all.
    - Grade 1: Moving with no forward progression, immotile. Also called grade D.
    - Grade 2: Moving with slow and wandering movement, not forward. (Non-progressive motility). Also called grade C.
    - Grade 3: Moving rapidly forward but in a curved line (non-linear motility). Also called grade B.

- Grade 4: Moving with high speed in straight line (progressive motility). Also called grade A.

10. A motility score is calculated by adding up the product motility grade and % of spermatozoa in that grade. The motility score for normal spermatozoa is ≥ 150.

21.4 REPORTING

The semen analysis is reported as follows:

- Aspermia — No ejaculate, absence of semen
- Hypospermia — Reduce semen volume
- Hyperspermia — Increase semen volume
- Azoospermia — Semen without sperms
- Oligozoospermia — Low sperms count
- Polyzoospermia — High sperms count
- Leucocytospermia — Increased number of WBCs
- Teratozoospermia — Morphologically abnormal sperm forms (>85%)
- Asthenozoospermia — Reduce or poor sperm motility
- Oligoasthenozoospermia — Low count with poor motility
- Necrozoospermia — Dead sperm

22. URINE ANALYSIS

The oldest known test on body fluids was done on urine in ancient times (before 400 BC). Urine was poured on the ground and observed to see whether it attracted insects. If it did, patients were diagnosed with boils. Hippocrates advocated a diagnostic protocol that included tasting the patient's urine, listening to the lungs, and observing skin colour and other outward appearances. Hippocrates related the appearance of bubbles on the surface of urine specimens to kidney disease and chronic illness.

Now urine is a valuable index to many normal and pathological mechanisms. The analysis can reveal conditions that are often unnoticed because they do not show striking signs or symptoms, e.g. diabetes mellitus, some types of glomerulonephritis, and chronic UTIs (urinary tract infections). The urine examination is performed by using urine test strips which indicate results as colour change. Another method is light microscopy of the urine samples. Urine is 94-96% water and remaining is made up of organic (urea, uric acid, amino acids, etc.) and inorganic material (sodium, potassium, chlorides and phosphates, etc.)

22.1 COLLECTION OF URINE SPECIMEN

For routine urine examination, the specimen is collected in dry and clean container. For microbiological examination, a midstream specimen is collected in a sterile container (to avoid debris and prostatic secretions). The sterile container can be prepared in the laboratory by sterilizing it in hot air oven at 160^0C for 1 hour. The container should be made up of glass with a metal screw cap. It is better to collect the specimen by catheterization in case of culture. Usually the female patient is asked to clean the external genitalia before collection. Although prior cleaning of the external genitalia is recommended, it has no proven advantage. Urine should be examined within 2 hours and if it cannot be examined promptly, must be refrigerated.

22.2 PRESERVATION OF URINE SPECIMEN

It is better to examine fresh specimen because delay in testing may result in some undesirable changes, which affect the test results. If there is a delay in examination, the urine should be refrigerated because it provides bacteriostatic

temperature (2-6^{0}C). When bacterial growth is not there, urea and glucose in urine are unaffected and thus no change in pH. Various chemicals are used to preserve the urine specimen and are known as preservatives, e.g. formalin, toluene, HCI, boric acid and thymol, etc. This preservation is very important to prevent growth of bacteria, preserving quantity of solutes, and preserving morphology of formed elements.

22.3 TYPES OF URINE SPECIMEN

Various types of urine specimens are collected for a variety of tests:

22.3.1 First Morning Specimen

This specimen is collected early in the morning before breakfast. It is more concentrated and best for nitrate and protein. It contains highest amount of HCG and therefore, is recommended for pregnancy test. It has acidic pH at which formed elements like red cells, white cells, epithelial cells, and casts are stable. This sample is not suitable for glucose testing.

22.3.2 Random Specimen

It is the sample collected at any time after the first morning sample. It is good for physical and chemical analysis.

22.3.3 Post-Prandial Specimen

It is collected usually after 2 hours of meal and best for sugar testing.

22.3.4 Afternoon Specimen

This sample is collected in the afternoon (2-4 pm). It is best for urobilinogen.

22.3.5 12 h Day Specimen

This sample is collected from 8:00 am in the morning to 8:00 pm in the evening. The urine is passed at 8:00 am and discarded. Then the entire specimen during the day is collected till 8:00 pm. Urine passed at 8:00 pm must be included in the lot.

22.3.6 12 h Night Specimen

This sample is collected from 8:00 pm in the evening to 8:00am in the morning. Urine passed at 8:00 pm is discarded and the entire specimen during the night is then collected till 8:00 am. The urine passed at 8:00am must be included in the lot. 12 hour day and night specimens are used for Addis's Sediment Count.

22.3.7 24 h Specimen

This sample is collected between 8:00 am of one morning and 8:00 am of next morning. The urine passed at 8:00 am is discarded and then the entire specimen is collected till next 8:00 am. The urine passed at 8:00 am must be included in the lot. 24 h specimen is required for the quantitative analysis of urinary proteins, sugars, electrolytes, hormones, urea, uric acid, and creatinine, etc.

22.4 PHYSICAL EXAMINATION

22.4.1 Appearance

Normally urine is clear or transparent. It may become cloudy on standing because of amorphous phosphates, urates, oxalates, pus, bacteria and fat, etc.

22.4.2 Volume

Normal 24 hours urine volume is about 1000 - 1800 ml and it depends upon age, fluid intake and weather. In an adult, night to day ratio is 1:2 to 1:3. When more than 3000 ml urine is excreted in 24 hours urine, it is termed as Polyuria and occurs due to excessive fluid intake, diuretics, diabetes mellitus, diabetes insipidus and hyper parathyroidism. When less than 500 ml and more than 100 ml urine is excreted in 24 hours, it is termed as Oligouria. This occurs as pre-renal like dehydration, shock, hemorrhage, renal like renal lethiasis, acute glomerulonephritis, and post-renal like urinary tract infections. Absence of urine or very little volume up to 100 ml is termed as Anuria.

22.4.3 Odour

Normal urine smells slightly aromatic. In the presence of acetone, e.g. in diabetes mellitus the odour becomes fruity. Bacterial decomposition of urine causes ammoniacal smell. Maple syrup like odour occurs either in the presence of pus or contamination with feces. Certain foods and medications also impart their smell to urine. A mousy odour is present in phenyl ketonuria.

22.4.4 Colour

Normal colour of urine is pale yellow because of presence of pigments called urochromes. These come from the metabolism of cytochromes, myoglobin, catalase and peroxidase. The colour varies with specific gravity. The colour of urine not only changes in certain diseases but also with the ingestion of foods, food dyes and medications. The colour is greenish orange in Jaundice due to bilirubin. It becomes smokey because of red blood cells and is light green in

diabetes mellitus. A black coloured urine is seen in alkaptonuria due to melanin or homogentisic acid.

22.4.5 Specific Gravity

Specific gravity provides information about ability of the kidney to dilute and concentrate urine. If the urine is concentrated the specific gravity will be high and if it is diluted, the specific gravity will be low. The specific gravity of urine varies directly with the grams of solute excreted per liter. The specific gravity of water is 1.000 and normal range for 24 hours urine varies between 1.015-1.025. The specific gravity of random sample is 1.003-1.032 and has no value. The specific gravity can be determined by Urinometer or Refractometer. With each 1% protein in urine, the specific gravity increases by 0.003 while for each 1% for glucose it increases by 0.004. In specimens containing these substances, the specific gravity should be corrected accordingly. When specific gravity is above normal, it is termed as hyperesthenuria and may occur in decreased intake of fluids, fever, dehydration, etc. When specific gravity is less than normal, it is termed as hypoesthenuria which occurs in increased fluid intake, hypothermia and sickle cell anaemia. Specific gravity is the ratio of weight of a volume of a substance to the weight of the same volume of the reference substance which is usually water.

22.4.6 Osmolalitv

The determination of urine osmolality is more important than specific gravity. This is due to the fact that it gives more accurate reflection of the concentration of dissolved substances than the specific gravity. The osmolality is a measure of the moles of dissolved particles contained in a kilogram of solvent. It reflects the total concentration of solutes. Normal urine osmolality is 40-1350 m osmol/kg (random sample).

22.4.7 pH

pH of the urine is the measure of hydrogen ion concentration of the urine. Urinary pH can vary from 4.5 to 8 but usually is a little acidic (5.5 to 6.5) because of metabolic activity. The acidic reaction of urine is due to the presence of urea, uric acid and creatinine, etc. Urine may become alkaline due to bacterial contamination. pH measurement can only be done on freshly voided urine. pH is measured with indicator paper strips and a colour chart for comparison. When exact estimation is required, a pH meter is used.

22.5 CHEMICAL EXAMINATION

In chemical examination, different types of chemicals and reducing substances are checked or examined in patient's urine. These include proteins, glucose, reducing sugars, ketone bodies, bile pigments, calcium, melanin, chloride, homogentisic acid, and amino acid.

22.6 MICROSCOPIC EXAMINATION OF URINE

The urine is microscopically examined for cells, casts and crystals. For the microscopic examination, urine is centrifuged at 2,000 rpm for 5 minutes. The supernatant is poured off and by flicking the end of tube with finger, re-suspend in a few drops of urine left. This is placed on the slide and examined. Staining of urine sediment can also be done. This makes possible the recognition of cells, particularly for the inexperienced technicians.

22.6.1 CELLS

a) Epithelial Cells

These vary in size and shape. Squamous epithelial cells are large cells with round or oval nuclei. These are normally present in small numbers. In females, very large number indicates vaginal contamination. They also indicate inflammation of urinary tract. Tubular epithelial cells appear in renal diseases.

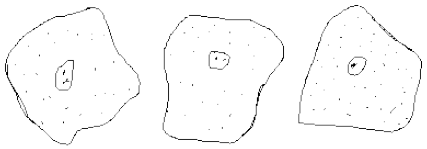


Fig 22.1 Squamous Epithelial Cells

b) Leucocytes (WBCs)

These are round granular cells, 10-15 u in diameter. They are found in clumps in UTI's. Normal urine from males does not contain more than 1 leucocyte per high power field (HPF), while from females it contains 1-5 per HPF. These are usually polymorphs and may show amoeboid movements in fresh specimen. Increased number, i.e. pyuria (pus in urine) indicates inflammation.

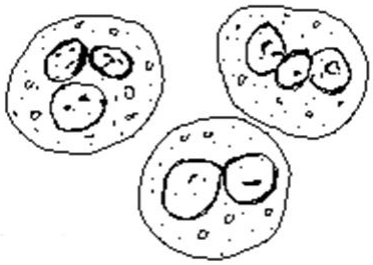


Fig 22.2 White blood cells

c) Erythrocytes (RBCs)

These appear as highly refractile, round, yellowish structures. These may be crenated and smaller in case of hypertonic urine. On the other hand, these are swollen and larger in case of hypotonic urine. Normal urine from males does not contain any RBC except if the specimen is collected by catheterization. Urine from females may show a few RBCs from vaginal contamination or many during menstruation. Except these two conditions, the presence is very critical and indicates bleeding in some part of urinary tract (haematuria). According to the American Urological Association, the occurrence of three or more red blood cells per HPF in two out of three urine specimens is the normally accepted definition of haematuria.

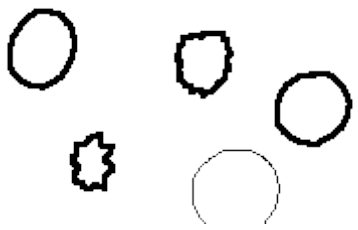


Fig 22.3 Red blood cells

22.6.2 CASTS

These are formed by solidification of protein (Tamm-Horsfall Protein) in the nephron tubule and are cylindrical in shape because they are formed in the tubules. Their size and shape depends on tubules where they were formed. They indicate widespread kidney disease. Casts are seen in increased number in urinary stasis, increased acidity, increased solute concentration, etc.

a) Hyaline Casts

These casts are cylindrical and transparent. They are usually colourless and normally found after heavy exercise. They are also seen in some diseased conditions such as renal failure, glomerulonephritis, cardiac failure, and diabetic nephropathy.

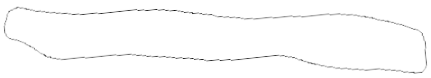


Fig 22.4 Hyaline cast

b) Finely Granular Casts

These casts are comparable to hyaline casts but have fine granules.

c) Coarsely Granular Casts

These have larger granules than the finely granular casts but are similar in appearance. The granules are degenerated cells or protein aggregates. The granular casts appear in nephrotic syndrome, pyelonephritis, and glomerulonephritis.

Fig 22.5 Granular cast

d) Leucocyte Casts

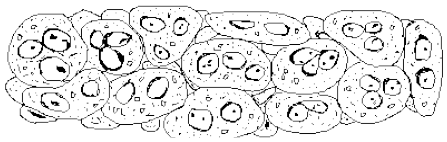


Fig 22.6 Leucocyte cast

These are composed of leucocytes. The leucocytes may be mixed with red cells or epithelial cells. Clumps of leucocytes may sometimes look like casts. These mainly appear in interstitial nephritis, lupus nephritis and acute pyelonephritis, etc.

e) Erythrocyte Casts

Fig 22.7 Erythrocyte cast

These are composed of red blood cells and frequently show a bright orange colour. These appear in acute glomerulonephritis, lupus nephritis, bacterial endocarditis and renal infarction, etc.

f) Haemoglobin Casts

These are brown and are formed either due to the presence of haemoglobin in the cast or due to degeneration of a red cell cast.

g) Fatty Casts

These casts contain fat droplets which are highly refractile.

h) Waxy Casts

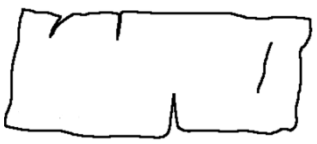


Fig 22.8 Waxy cast

These are comparable to hyaline casts, however, these are opaque and usually have a curled tail. Some pathological conditions in which these are found are severe tubular atrophy, renal failure and transplant rejection, etc.

i) Cylindroids

Cylindroids are long ribbon like structures resembling hyaline casts. The only difference is that these are longer and often tapered.

22.6.3 CRYSTALS

These are not seen in fresh warm urine but formed after some time. Except for cystine, uric acid, leucine and tyrosine crystals, they have very little significance. The types of crystals present are dependent on pH of the urine.

a) Calcium Oxalate

Fig 22.9 Oxalate crystals

These are colourless, octahedral crystals and appear as small squares crossed by two diagonal lines. They vary greatly in size.

b) Uric Acid

Uric acid crystals appear in the form of plates, prisms, sheaves, and hexagons. The crystals are usually coloured (yellow or reddish brown). They are soluble in NaOH but insoluble in HCI.

c) Amorphous Urates

Urates often appear in the form of amorphous sediment which dissolves on heating. Sodium urate crystals are often seen in the form of thorn apples. The colour is yellow or reddish brown. These are called amorphous phosphates if appear in alkaline urine.

d) Cystine

These are highly refractile hexagonal plates, similar to the plate form of uric acid crystals. However, they may be differentiated from uric acid crystals by their solubility in HCI. They are indicative of cystinuria.

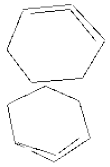


Fig 22.10 Cvstine crystals

e) Tyrosine

These crystals are seen in liver failure and have yellow colour. They appear as fine needles or sheaves.

f) Leucine

These are yellow, oily appearing spheres with radial and concentric striations. Their presence is related to liver failure.

g) Cholesterol

These appear as rectangles with cut corners are found in severe renal damage or when a lymphatic vessel ruptures in renal pelvis.

h) Alkaline Urine Crystals

These include amorphous phosphates, triple phosphate, calcium carbonate and ammonium urate. In addition, due to administration of some drugs crystals may appear such as sulphonamide crystals and ampicillin crystals.

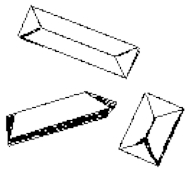


Fig 22.11 Triple phosphate crystals

23. STOOL ANALYSIS

This analysis is performed for parasites, ova and cysts especially:
-Entamoeba histolytica -Giardia intestinalis -Ascaris lumbricoides
-Trichuris trichiura -Taenia species - Hymenolepis species
-Enterobius vermicularis, etc.

23.1 MACROSCOPIC EXAMINATION

The stool is macroscopically tested for:

- Colour Brown
- Consistency Formed/Semi-formed
- Mucus Absent
- Reducing substances Absent
- Blood Absent

23.1.1 Reducing Substances in Stool

This is used to detect the reducing sugars in stool. The test used is called Benedict's Test and performed as follows:

1. Take 5 ml Benedict's reagent in a glass test tube.
2. Add 10 drops of fluid faeces (faeces + normal saline).
3. Keep test tube in boiling water or on a flame until it is boiled.
4. Observe for a colour change according to below scheme:

Colour	**Result**
Blue green	Negative
Yellowish green	Traces
Yellow	+
Yellow with heavy precipitates	++
Brown with precipitates	+++
Brick Red	++++

23.1.2 Occult Blood Examination in Stool

This examination is performed for the detection of lyzed red blood cells in stool and performed as follows:

1. With an applicator, prepare a very thin smear of stool inside area A and B of slide.
2. Open perforated section on the back of slide (opposite side of stool sample) and add a few drops of hema-screen developer (stabilized solution of hydrogen peroxide and denatured alcohol).
3. Observe the result after 30 seconds.
4. Compare result with the control (blue colour).
5. Blue colour in test section will indicate the presence of blood in stool sample.
6. If there is no blue colour, the result is negative.

23.2 MICROSCOPIC EXAMINATION

Stool routine microscopic examination is performed by Direct and Formol-Ether Concentration techniques.

23.2.1 Direct Method for Stool Examination

1. The slide is labeled and a drop of normal saline is placed.
2. A drop of iodine is placed on the same slide. Mix a small amount of stool with wooden stick in normal saline as well as in iodine to make a suspension which is neither too thick nor too thin.
3. Cover each drop with a cover slip.
4. Examine both preparations under the microscope.
5. Place cover slip and observe the smear microscopically, first with low power objective followed by examining with the high power objective lens to look for motile flagellates any ova or cysts. It is important that condenser iris is fully open when using oil immersion lens.

23.2.2 Formol-Ether Concentration Method for Stool

1. Pick about 2 ml (2 gm) of stool sample and mix well in 10 ml of saline solution.
2. Filter the suspension through two layers of gauze into a centrifuge tube. (An ordinary tea or coffee strainer made of fine mesh can be used instead of gauze and can be washed and reused for other samples).
3. Centrifuge the filtrate for 1 minute at a medium speed.
4. Discard the supernatant. If the supernatant is very cloudy, wash the deposit again with 10 ml saline and re-centrifuge. Pour off the supernatant.
5. Add formaldehyde reagent to the deposit up to 10 ml.

6. Shake the mixture vigorously and let stand for 5 minutes.
7. Add 3 ml of ether or petrol (up to the 3 ml mark). Care must be taken that there should be no open flame in the laboratory.
8. Properly cap and shake vigorously for 30 seconds.
9. Centrifuge for 1 min at low speed.
10. Tube will show four layers starting from the top; (1) Ether (2) Debris (3) Formaldehyde and (4) Deposit at the bottom containing the cyst and ova.
11. By using a wooden applicator, free the layers of the debris from the side of the tubes. Pour off all the supernatant fluid.
12. Mix the small amount of remaining fluid well with the deposit.
13. Add 2 drops of Lugol's iodine in deposits and mix it well.
14. Observe for ova and cyst under the microscope (40x).

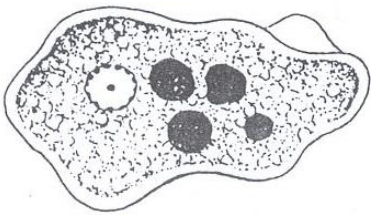


Fig 23.1 Entamoeba histolytica trophozoite

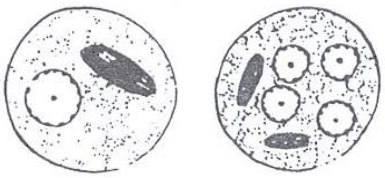


Fig 23.2 Entamoeba histolytica cyst

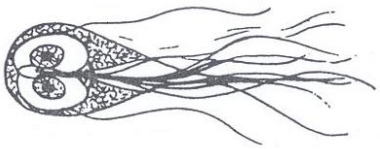


Fig 23.3 Giardia Lamblia trophozoite

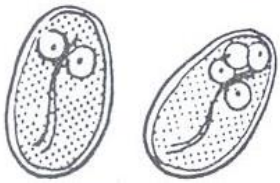
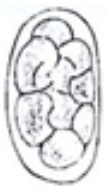


Fig 23.4 Giardia Lamblia cyst

Table 23.1 Characteristics of Eggs of Commonly Seen Helminths in Stool

Helminths	Size	Shape	Shell	Contents	Appearance
Ascaris lumbricoides (round worm) Fertilized egg with double shell	70 um	Oval, occasionally round	External layer rough brown and mammillated while internal layer is thicker, smooth and colourless	A single round granular central mass	
Ascaris lumbricoides (round worm) Unfertilized egg with double shell	80-90 um	More elongated	Brown and puffy. Two layers are indistinct	Full of large round refractile granules	
Ankylostoma duadenale (hook worm)	50-60 um	Oval, rounded, slightly flattened poles	Very thin, similar to black line	4-16 grey granular cells (fresh stool) A small larva wrapped around itself may fill the egg in old stool (12-48 hours)	
Enterobius vermicularis (thread worm)	50-60 um	Oval but asymmetrical (flattened on one side and rounded on the other)	Smooth and thin, a double line is visible	A small granular mass or a curled up larva	

24. DISTINGUISHING FEATURES OF COMMON PATHOGENS

Staphylococcus aureus

Characteristics	Gram positive cocci arranged in grapelike clusters, facultative, coagulase positive, catalase positive
Habitat and Transmission	Human nose and skin, transmission via hands
Diseases	Abscesses in various organs, sepsis, wound infection, endocarditis, food poisoning, toxic shock syndrome
Laboratory Diagnosis	Gram staining and culture. On blood agar, 1-3 mm golden yellow colonies, often haemolytic, coagulase positive

Streptococcus pyogenes

Characteristics	Gram positive cocci in chains, catalase negative
Habitat and Transmission	Human throat and skin, transmission via respiratory droplets
Diseases	Suppurative – Pharyngitis, cellulitis, others Non-suppurative (long term sequela) – Rheumatic fever, rheumatic heart disease, acute glomerulonephritis
Laboratory Diagnosis	On sheep blood agar, small 1mm beta haemolytic colonies, bacitracin sensitive (0.04 U), catalase negative

Streptococcus pneumoniae (Pneumococcus)

Characteristics	Gram positive lancet shaped diplococcic lying end to end, capsulated, alpha haemolytic, catalase negative
Habitat and Transmission	Human respiratory tract, transmission via respiratory droplets

Diseases	Lobar pneumonia, meningitis, otitis media
Laboratory Diagnosis	Gram stain and culture, small alpha haemolytic colonies on blood agar, draughtman colonies after further incubation, optochin sensitive

Enterococcus (S. faecalis)

Characteristics	Gram positive cocci ovoid forms in pairs or short chains, somewhat larger than S. pyogenes, catalase negative
Habitat and Transmission	Human colon, faecal oral transmission
Diseases	Urinary tract infections, sepsis, endocarditis
Laboratory Diagnosis	Culture, grows on ordinary media, unlike other streptococci grows on MacConkey to form 0.5-1 mm pink lactose fermenting colonies, also grows at $45^{O}C$ and in 6.5% NaCl (agar or broth). On blood agar, produces alpha, beta or no haemolysis

Neisseria gonorrhoeae and Neisseria meningitidis

Characteristics	Gram negative kidney shaped diplococcic, oxidase positive
Habitat and Transmission	Upper respiratory tract, transmission via respiratory droplets
Diseases	Meningitis, septicaemia, gonorrhoea
Laboratory Diagnosis	Gram stained smear shows gram negative intracellular diplococcic. Culture in chocolate agar in CO_2 oxidase positive small colonies

Salmonella typhi

Characteristics	Gram negative rods, motile, non-lactose fermenting, produce H_2S in TSI

Habitat	Human colon, faecal-oral transmission, exclusively human pathogens
Diseases	Typhoid fever
Laboratory Diagnosis	Blood culture, sub cultured and identified by biochemical and serological reactions. Widal test, Typhidot

Shigellae

Characteristics	Gram negative, non motile, non-lactose fermenting
Habitat	Human colon, faecal-oral transmission, exclusively human pathogens
Diseases	Enterocolitis (dysentery)
Laboratory Diagnosis	Stool culture, identified by biochemical and serological reaction

Vibrio cholerae

Characteristics	Comma shaped gram negative rods, darting motility, oxidase positive
Habitat	Human colon, faecal oral transmission
Diseases	Cholera
Laboratory Diagnosis	Stool – direct motility and gram stain, culture

Escherichia coli

Characteristics	Gram negative motile rods, lactose fermenting
Habitat	Human colon, lower genitourinary tract (normal flora)
Diseases	UTI (ascending infection), sepsis, neonatal meningitis
Laboratory Diagnosis	Culture, lactose fermenting colonies on MacConkey agar

Proteus

Characteristics	Gram negative rods, highly motile, non- lactose fermenting, urease positive
Habitat	human colon (normal flora), soil and water, UTI transmitted by ascending spread of faecal flora
Diseases	UTI, sepsis
Laboratory Diagnosis	Culture, swarming growth on blood agar, non-lactose fermenting colonies on MacConkey differentiating from Salmonella by urease production

Klebsiella pneumoniae

Characteristics	Gram negative capsulated rods, non-motile
Habitat	Human upper respiratory tract and GIT, transmitted via respiratory droplets, UTI transmission by ascending spread of faecal flora
Diseases	UTI, pneumonia, sepsis
Laboratory Diagnosis	Culture-highly mucoid colonies on blood agar, lactose fermenting colonies on MacConkey agar, confirmed by gram stain and motility (non-motile)

Pseudomonas aeruginosa

Characteristics	Gram negative motile rods, strict aerobe, oxidase positive, non-lactose fermenting on MacConkey agar, green pigment and a sweet smell on blood agar
Habitat	Environment, transmitted via water aerosols, aspiration, contact
Diseases	Wound infections, UTI, sepsis, pneumonia. One of the most important nosocomial pathogen, resistant to many antimicrobials
Laboratory Diagnosis	Culture-greenish pigment on blood agar and nutrient agar with a characteristic sweet smell, oxidase positive, non-lactose fermenting on MacConkey agar.

25. MICROBIOLOGICAL ANALYSIS OF WATER

Waterborne infections are the main cause of human morbidity and mortality in under developed countries. The main cause is the contamination of water supplies by human or animal excretions. If such a contamination is coming from cases or carriers of intestinal pathogens like Vibrio cholerae, Salmonella or Shigella, water may contain viable organisms and the use of such water can lead to the spread of infectious diseases. Also the use of this contaminated water for the preparation of food is very hazardous for human. Intestinal organisms are used as indicators of faecal contamination. In water having coli form bacteria in general and E. coli in particular, will be consider as an evidence of faecal contamination while that of E. coli will be a final proof.

25.1 COLLECTION AND TRANSPORT OF WATER SAMPLES

Samples should be collected into 150 ml screw capped sterile glass bottles. Screw cap the bottle after collection and cover neck of the bottle with a paper or aluminum foil.

25.2 METHODS OF WATER ANALYSIS

25.2.1 Total Count

It is also called colony count and standard plate count and gives the exact number of microorganism including bacteria, yeast and mould. It is usually done by pour plate method. In this method, a known volume of water sample is mixed with the agar in Petri dish and allowed to set.

25.2.2 Membrane Filtration Method

This method is applied when the number of microorganism is very low in water. The bacteria are filtered from a membrane. The bacteria along with the membrane are placed on an appropriate solid culture media. During incubation, these bacteria produce visible colonies that are counted.

25.2.3 Multiple Tube Dilution Method

This is also known as Most Probable Numbers or MPN method and based on the statistical values of the result obtained, can detect and estimates various groups of microorganisms in water sample. A series of tubes containing an appropriate selective broth culture media is inoculated with appropriate volumes of a water sample. After a specified incubation time and temperature, each tube presenting with acid and gas production is considered ‘Presumptive Positive’

since the gas signify the probable presence of coli form. However, other organisms can also produce gas, so a subsequent confirmatory test for faecal coli forms (E. coli) is mandatory. The two tests available in laboratories are the presumptive coli forms test and confirmatory test for faecal coli forms (E. coli). MacConkey broth (purple-Oxoid) with Durham tubes are used as culture media.

The confirmatory test for E. coli is carried out from each presumptive positive tube in the water bath at 44^{o}C for 24 hours and peptone water at 37^{o}C. Growth and gas production at 44^{o}C and a positive indole test shows the presence of E. coli. Growth and gas production in the absence of indole production indicates thermo tolerant coli forms.

25.3 INTERPRETATION

MPN are interpreted on the presence of acid and gas, while in total count and membrane filtration method the number of colonies are counted.

26. MICROBIOLOGICAL ANALYSIS OF FOOD

Microorganisms are linked with all the food we eat. They can affect the quality, availability and quantity of our food. The foods which are natural, e.g. fruits and vegetables normally have some microorganisms and can be contaminated with additional microorganisms through handling. Food act as a medium for the growth of microbes and this growth can result in the decomposition and spoilage of food. Food may also carry a pathogenic organism and may become a source of pathogenesis and as a result transmit diseases. So, the major concern in food microbiology is the control of microorganisms. Types of food tested in microbiology laboratory include meat, poultry, eggs, fruits, vegetables, shellfish and milk.

26.1 METHODS OF FOOD ANALYSIS

26.1.1 Direct Microscopy

Standard microscopic techniques are available for the examination of some food products, e.g. a procedure known as the bread smear is used to make a direct microscopic count of microorganisms in milk. The essential procedure of this technique is (1) spreading a measured amount of milk over a known area on a glass slide; and (2) staining the film of milk with methylene blue.

26.1.2 Total Count (Differential Enumeration)

For total count, microscopic counts are made in several microscopic fields and the total number of bacteria per unit volume is counted.

26.1.3 Culture Techniques

Plate culture technique is used for the enumeration of the total microbial population or some particular group of microorganism. Standard plate count (SPC) are used for the enumeration of bacteria in milk, other cultures procedures are available for particular physiological or biochemical types of microorganisms. For the cultivation of viruses from food, tissue culture techniques are required.

SECTION-C VIROLOGY

27. INTRODUCTION

The branch of microbiology that deals with medically important viruses is called virology. Viruses are very small acellular organisms containing either single or double stranded DNA or RNA whereas bacteria contain both. The immune response is predominately cell mediated in case of viruses (antibody mediated in bacteria). Most of the viruses range in size from 20-30 nm in diameter and can only be seen through an electron microscope. The viruses are complex structures and their shape is determined by the capsid pattern (protein coat surrounding DNA or RNA). This pattern is also used in viral classification, i.e. it can be Icosahedral (cubic) in which capsomeres are seen in 20 triangles, forming shape of a sphere. It can also be Helical where capsomeres appear rod-shaped. Viruses are also classified on the basis of genetic material, i.e. DNA viruses and RNA viruses.

Viruses do not have cellular structures and organelles; therefore, they cannot form essential proteins. Due to this reason, they are completely dependent on host cells for survival. Viruses are inactive outside living cells. There are proteins on capsid and have several important functions, e.g. attaching the virus to host cells, protecting the genetic material of virus, etc. Some viruses have an envelope on their outer surface. If the virus does not have an envelope, it is called, Naked Virus. When an infection is caused by a virus, the host cells dies and the effect of virus which leads to cell death is called cytopathic effect (CPE).

27.1 VIRAL INFECTIONS AND PAKISTAN

For the last few years, incidents of multiple viral infections are increasing in Pakistan. Polio and Hepatitis infections are at higher index and Dengue outbreaks in the recent past became the real threat and catastrophe. It has become an annual recurring event in Pakistan and it is now evident that Dengue has become a regular epidemic phenomenon in Pakistan. Dengue also is presently amongst the most important arthropod-borne infections from both the medical and public health perspectives. These infections leave irreversible damaging effects on socio-economic aspect of the country. The magnitude of these viral infections is one of the major financial burdens on Pakistan

economy. Major viral diseases in Pakistan include Polio, Viral Hepatitis, HIV, Influenza (H1N1) and Dengue. Pakistan is among three countries where polio is still categorized as an endemic viral infection. Pakistan persists with the existence of all forms of viral hepatitis (A, B, C, D, E) with a high prevalence rate. If we narrow the spectrum, hepatitis C and hepatitis B are with higher prevalence index with 7.4%. HIV/AIDS is also one of the major viral infections with increase incident rate in Pakistan. According to NACP (National AIDS Control Programme) 97,400 individuals are infected with this deadly disease. The officially reported cases are much lower and only show tip of the iceberg. If the proportion of people living in poverty is to be lastingly reduced, integrated and sustainable measures to reduce infectious diseases at the country level are indispensable.

28. LABORATORY DIAGNOSIS OF VIRUSES

Diagnostic science has evolved multiple methods to diagnose viruses with times. The following five approaches among those are used for the diagnosis of viral diseases from clinical specimens:

28.1 IDENTIFICATION FROM CELL CULTURE

All viruses require cell culture(s) to grow because viruses replicate only in living cells and not on cell-free media. Since many viruses are inactivated at room temperature, hence, it is important to inoculate the specimen into the cell culture at earliest possible convenience. Viral growth in cell culture often produces a characteristic cytopathic effect (CPE) that can provide the basis of probable identification. The time taken by the virus for CPE and the type of cell in which the virus produces the CPE are critical indicators in the presumptive identification.

If a virus fails to produce any CPE, its presence can be detected by several other techniques:

1. Haemadsorption, i.e. attachment of RBCs to the surface of virus-infected cells.
2. Interference with the formation of a CPE by a second virus, e.g. rubella virus which does not cause a CPE, can be detected by interference with the formation of a CPE by certain enteroviruses.

Colour change in the phenol red culture medium due to decreased acid production by infected and dying cells could also be used as a useful tool for virus identification. This change could be detected by identification of virus by using known (specific) antibody. This method is used in several tests which are:

- Complement Fixation Test (CFTs).
- Haemagglutination Inhibition (HAI)
- Neutralization
- Fluorescent-Antibody Assay
- Radioimmunoassay (RIA)
- Enzyme Linked Immunosorbent Assay (ELISA)
- Immunoelectron Microscopy

28.2 MICROSCOPIC IDENTIFICATION

Viruses can be detected and identified by direct microscopical examination of clinical specimens such as biopsy material or skin lesions.
Three different procedures can be used:

- Light microscopy reveals distinctive inclusion bodies and/or multi-nucleated giant cells. Although this is an indirect technique, it can lead a virologist to identify a virus.
- Ultraviolet (UV) microscopy used to detect fluorescent antibodies of the virus in the infected cells. Again an indirect method but with better specificity than light microscope.
- Electron microscopy detects direct viruses which can be classified by their size and morphology. Different stains could also be used for better illustrations such as osmium tetraoxide, phosphotungstic acid, etc.

28.3 SEROLOGICAL (IMMUNOLOGICAL) PROCEDURES

In this approach, a rise in the titre of specific antibody against particular virus can be used to diagnose current infection. A plasma or serum sample is obtained as soon as a viral etiology is suspected (acute phase) and secondary sample is obtained 10-14 days later of onset of infection (convalescent phase). If the convalescent phase (serum sample) shows at least 4-fold higher antibody titre than that of the acute-phase (serum sample), the patient is considered to be infected. The antibody titre can be determined by many of the immunological tests already mentioned above. In hepatitis B viral disease, the presence of IgM (acute phase) against HBcAg (Hepatitis B Core Antigen) indicates acute Hepatitis B infection. Other nonspecific serological tests are also available, e.g. heterophil antibody test for infectious mononucleosis.

28.4 VIRAL ANTIGENS DETECTION

A variety of viral antigens can be detected in patient's blood and or body fluids by various techniques but ELISA and its advanced forms (MEIA, CMIA) are common. Tests for the surface antigen of HBV, the P24 antigen of HIV are among the common examples of this method. Further techniques used for this method are;

1. Radio Immunoassay (RIA)
2. Microparticle Enzyme Immunoassay (MEIA)
3. Fluorescence Polarization Immunoassay (FPIA)

4. Chemiluminescence Microparticle Immunoassay (CMIA)
5. Electro-Chemiluminescence Immunoassay (ECLIA)

28.5 DETECTION OF NUCLEIC ACIDS

Detection of viral nucleic acids either DNA or RN,A is the most specific method for diagnosis of viral infections. Viral nucleic acids can be obtained from multiple specimens like blood, body fluids, stool, sputum and tissues. PCR (Polymerase Chain Reaction) is the technique of choice for this purpose because it amplifies the viral nucleic acids and detection becomes easy. HCV RNA detection from blood sample and Mycobacterium Tuberculosis (MTB) DNA detection from bronchoalveolar lavage or sputum are main examples of this method. PCR technique is used to monitor the anti-viral therapy response by detecting the viral load either copies or IU per milli litres of blood. Nucleic Acid Testing (NAT) is the upgraded form of PCR used in blood transfusion to screen the infectious diseases at a molecular level.

29. COLLECTION, STORAGE AND TRANSPORTATION OF SPECIMEN FOR VIROLOGICAL EXAMINATION

Sample selection, collection and subsequently transportation are the vital steps in any diagnosis process of a viral infection. An adequate specimen collected after following aseptic measures and good laboratory practices from an appropriate site could lead to better diagnosis. Similarly the proper provisional storage of the specimen followed by prompt transportation to the laboratory also helps a lot in the final diagnosis.

Since different types of samples are required to diagnose viruses in different viral diseases so the strategy to collect and store those samples differs from sample to sample. Specimens must be collected in the acute phase of disease. Herpes Simplex Virus (HSV) and Varicella-Zoster virus might not be retrieved from lesions after 5 days of onset of clinical manifestations of disease. Similarly respiratory viruses could remain viable during 3-7 days viral shedding period following infection. Isolation of an enterovirus (Coxsackie virus, Echovirus) from the CSF is most helpful within 2-3 days after onset of the symptoms of infection in CNS.

29.1 MAJOR GUIDELINES

1. Most viral specimens should be stored at 2-8°C rather than frozen for short term (<48 hours) transit and storage.
2. For delays exceeding 48 hours, freeze viral specimens at -70°C or below. Do not freeze at -20°C since DNA/RNA remain viable below -70°C.
3. Sterile body fluids such as CSF do not require any transport medium and should not be diluted.
4. Use of commercially available transport media is preferred as an immediate alternative to in-house transport media for swabs (eye/ear/pus, etc) and washings (bronchial, etc).
5. Swabs with calcium alginate must be avoided while collecting specimens for HSV and Chlamydia cultures. The fibers present within media may inactivate these viral agents.
6. Use of wooden shaft swabs should also be avoided because these can activate certain inhibitory process into viruses.

7. It is usually not possible to isolate Arboviruses from clinical specimens. In such cases serological and immunological methods are useful.
8. Hepatitis viruses (HAV, HBV, HCV, HEV, etc) are best detected from blood and serum/plasma could be stored at -70°C if there is delay in testing.

29.2 TYPE OF VIRAL TRANSPORT MEDIA (VTM)

There are many viral transport media easily available in the market. It is better to use these commercially available media rather custom preparations. WHO has commercialized viral transport media marketed as COPAN Universal Transport Medium. Eagle Minimum Essential Medium (E-MEM) is another suitable commercially available medium for this purpose. Companies dealing in pre-analytical solutions also launched their patent VTM like "BD™ Universal Viral Transport System" which is a complete package for sample storage and transit. Alternatively, VTM could also be prepared in house as under:

- Add 10g veal infusion broth and 2g bovine albumin fraction V to 400 ml sterile distilled water.
- Add 0.8 ml gentamycin sulfate solution (50 mg/ml) and 3.2 ml amphotericin B (250 µg/ml)
- Gently mix the solution and sterilize by filtration technique and VTM is ready to use.
- This medium is a suitable VTM for use in collecting throat and nasal swabs.

Following table describes in detail the type of samples, its storage and transportation:

Specimen Source Procedure	Collection Procedure	Optimum Transport
Blood	Collect 1 tube (4-7 mL) of Heparinized (green top) or EDTA (purple top) blood	Room Temperature
Body fluids other than blood or urine	Collect 2-3 mL in a sterile container	Up to 48 hours at 2-8° C (Refrigerated) Over 48 hours at -70° C (Frozen)
Bone marrow	Collect 2 mL in Heparin or EDTA	Up to 48 hours at 2-8° C (Refrigerated) Over 48 hours at -70° C (Frozen)
Bronchial wash/brush or	Collect 2-3 mL and place in viral transport medium	Up to 48 hours at 2-8° C (Refrigerated)

alveolar lavages		Over 48 hours at -70° C (Frozen)
CSF	Collect 1mL in a sterile container. Do not dilute in VTM	Up to 48 hours at 2-8° C (Refrigerated) Over 48 hours at -70° C (Frozen)
Eye swab or scraping	Swab the inflamed conjunctiva or corneal lesions. Place swabs or scrapings in viral transport medium.	Up to 48 hours at 2-8° C (Refrigerated) Over 48 hours at -70° C (Frozen)
Nasopharyngeal	Collect 2 nasopharyngeal swabs. Place both swabs in viral transport medium.	Up to 48 hours at 2-8° C (Refrigerated) Over 48 hours at -70° C (Frozen)
Sputum	Collect in a sterile container	Up to 48 hours at 2-8° C (Refrigerated) Over 48 hours at -70° C (Frozen)
Stool	Collect 1-2 grams of fresh stool	Up to 48 hours at 2-8° C (Refrigerated) Over 48 hours at -70° C (Frozen)
Swab	Collect on sterile swab and place in viral transport medium. Do not use wooden shafted swabs or calcium alginate swabs.	Up to 48 hours at 2-8° C (Refrigerated) Over 48 hours at -70° C (Frozen)
Throat	Collect on sterile swab and place in viral transport medium.	Up to 48 hours at 2-8° C (Refrigerated) Over 48 hours at -70° C (Frozen)
Tissue	Place in viral transport medium.	Up to 48 hours at 2-8° C (Refrigerated) Over 48 hours at -70° C (Frozen)
Urine	Collect 5 mL in a sterile container.	Up to 48 hours at 2-8° C (Refrigerated) Over 48 hours at -70° C (Frozen)
Vesicular lesion	Collect the fluid and cellular material from the base of several fresh vesicles. Place in viral transport medium. Do not use calcium alginate swabs or swabs with wooden shafts.	Up to 48 hours at 2-8° C (Refrigerated) Over 48 hours at -70° C (Frozen)

30. LABORATORY DIAGNOSIS OF HIV, HBV AND HCV

30.1 LABORATORY DIAGNOSIS OF HIV/AIDS

The Acquired Immunodeficiency Syndrome (AIDS) is a viral disease caused by Human Immunodeficiency Virus (HIV). The HIV is an RNA reverse transcribing virus belonging to lentivirus subgroup of retroviruses that cause slow infections with long incubation periods. The virus transfers from one person to another through unscreened blood transfusion, contaminated hypodermic needles and sexual relationship. Additionally, infected pregnant women can pass HIV to their babies during pregnancy, delivery, and also through breast feeding. More than 25 million people have died of AIDS since its discovery in 1981, affects 0.6% of the world's population with 2.5 million cases arising every year. Pakistan is a developing country with 190 million people living in low health and educational standards. It is the second largest country in South Asia that stands only a few steps behind India and Nepal in terms of HIV epidemic. In Pakistan, the first case of AIDS was reported in 1987 and since then the number has increased dramatically. According to National AIDS Control Programme, there are about 97,400 people living with HIV in the country, however, the actual data proposes almost half of that number.

The diagnosis of HIV is very important in order to avoid further transmission of infection. The laboratory diagnosis of HIV infection is primarily based on antibody screening tests such as Enzyme Linked Immunosorbent Assay (ELISA) and rapid tests. In addition to the antibody screening assays, confirmatory testing with Western blot is also performed. For monitoring of patients on antiretroviral therapy and for HIV detection in newborns, Polymerase Chain Reaction (PCR) is used. The main objectives of HIV antibody testing are; (1) Transfusion, transplant or donation safety; (2) Surveillance of HIV infection; and (3) Diagnosis of HIV infection. No single test satisfies all conditions or objectives. Different objectives require specific strategies (combination and sequence of tests), while local conditions such as daily volume of tests, staff training levels and comparative costs influence the type of test chosen. Assays for HIV differ in methodology as well as testing purpose. All screening tests detect all known HIV types (HIV-1 and -2), HIV groups and HIV subtypes. Based on antigen-antibody reactions, HIV antibody tests are the most appropriate for routine HIV diagnosis. There should not be

any HIV testing without voluntary counseling and education. UNAIDS and WHO have published several recommendations and guidelines for HIV antibody testing but the commonest algorithm is a sequential two-test algorithm, which consists of an ELISA screening test and re-testing of reactive samples by Western blot assay as a supplemental test, to confirm HIV positivity.

30.2 LABORATORY DIAGNOSIS OF HEPATITIS B VIRUS

HBV belongs to a family of hepadnaviridae virus family; structurally this is icosahedral particle in which the core is composed of protein while the genome is composed of DNA. In Pakistan, 2.5% people are affected by this deadly disease. There are different antigens of the virus which are used for diagnostic purposes. The examples are surface antigen (HBsAg), Core antigen (HBcAg), e antigen (HBeAg) and HbX protein.

The methods available for the diagnosis of HBV infection are Immuno-chromatography (the most primitive type of diagnostic procedure), ELISA (specific and more informative lab diagnostic technique and includes several sub parameters like Hepatitis B core IgM, HBeAb. HBsAg and HBeAg), and PCR which measures the concentration of hepatitis B viral DNA in patient serum. PCR enables the viral load at the beginning of treatment to be established and thereafter monitored to indicate treatment success. PCR can be qualitative and/or quantitative. In addition, HBV genotyping can be performed which identifies the hepatitis B genotype A to H in a patient's serum. This is critical in determining treatment and monitoring response. The HBV Drug Resistance detects a hepatitis B virus wild type and drug induced mutation associated with famciclovir and lamivudine drug resistance, including YMDD mutants. The detection of HBsAg for more than 6 months is indicative of chronic carrier state. The HBcAb is positive even during the window period.

30.3 LABORATORY DIAGNOSIS OF HEPATITIS C VIRUS

HCV is an RNA enveloped virus which causes infection the liver and is responsible for the chronic liver diseases. HCV belongs to the genus Hepacivirus and is member of the family Flaviviridae. It is the type of retro viruses which is composed of RNA and envelope. As the hereditary material is composed of RNA which is naked as compared to DNA, hence more prone to

mutation which is one of the major factor in failure of therapy. In Pakistan, 4.9% people are affected by this fatal disease.

HCV infection is diagnosed in laboratory by a variety of methods. Some of commonly practiced methods are Immuno-chromatography (which detects antibodies in the patient's serum non-specifically and qualitatively) and ELISA (detects anti HCV antibodies quantifiably). In case of HCV, there is no antigen assay which is usually more specific as compared to antibodies detection. The PCR is also performed and measures the concentration of hepatitis C viral RNA in patient serum. PCR can be qualitative and/or quantitative. In some cases, HCV genotyping is also performed. The identification of HCV genotypes is helpful in management of treatment.

31. POLYMERASE CHAIN REACTION (PCR)

PCR is a biochemical technique of molecular biology used for identification (qualitative) and quantification (quantitative) of nucleic acid either DNA or RNA in any specimen. This method was developed and introduced in 1983, by Kary Mullis and got Nobel Prize in 1993 for his premier invention. PCR now has multiple applied repercussions starting from disease diagnosis, paternity testing, forensic medicine and biological research. PCR has provided us the basis of modern age applications like DNA sequencing and cloning, gene analysis for its therapy and DNA microarray. PCR amplifies the specific region of nucleic acid to multiple copies during a series of repeated temperature change cycles in a thermocycler. A typical PCR includes following procedural steps:

1. **Denaturing:** DNA detached into 2 independent strands at 94-98°C for up to 30 seconds.
2. **Annealing:** Primer attached to single strand DNA at 50-65°C for 20-40 seconds.
3. **Extension/Elongation:** DNA strand extended by DNA Polymerase at 75-80°C.
4. **Cooling:** to get a final product at 4-15°C.

Following ingredients are required to carry out a PCR:

- **DNA template:** it contains specific DNA region to be amplified.
- **Primers:** they lead the extension, complementary to the 3' (three prime) ends.
- **Taq polymerase:** (DNA polymerase) an enzyme to extend DNA strand.
- **Deoxynucleoside triphosphates (dNTPs):** the building-blocks for a new DNA strand synthesis.
- **Buffer:** providing a medium for optimum activity.
- **Divalent cations:** magnesium or manganese ions.
- **Monovalent cation:** potassium ions.

Conventional PCR: It is the first and prime type. Impressions of amplified nucleic acids are taken by gel based electrophoresis in different bands. The bands are then identified according to the molecular weight which is compared with ladder marker.

Real Time PCR: It is advanced and quicker type in which amplification and identification of nucleic acid is carried out simultaneously through computer operated program by using light emitting probes (light attached primers).

32. ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA)

ELISA is an immunological method widely used in diagnostics to detect either antigen (Ag) or antibody (Ab) from a blood specimen. It could either be qualitative or semi-quantitative. It was developed as a safer alternative to radioimmunoassay (RIA) where there is a potential threat to the health of workers and environment by radiations. Its invention remained contradictory as there are many scientists who claimed for it but Jerker Porath was the first scientist who published the concept of this technique in 1966. In ELISA, the results are actually measured as an absorbance signal that is compared with that of a cutoff value; results above the cutoff are reported as positive, whereas those below the cutoff are called negative. It is based on colourimetric reactions of chromogenic substrates, (such as TMB) and label enzymes.

ELISA has provided us a useful way to measure either antigen or antibody concentration in two main ways:

a) It can be performed to detect the presence of antigens in a sample (liquid) by using specific antibody coated with the surface of well (stationary solid phase) or
b) It can be followed to assess the presence of antibodies in a sample (liquid) that recognize the antigen coated with the well surface (stationary solid phase).

A typical ELISA has six steps to proceed:

1) Take antigen coated microtiter plate wells.
2) It binds with specific antibody (at optimum temperature) when sample is added. (Ag/Ab complex)
3) Excess particles are eliminated by washing.
4) Enzyme conjugated with an antibody (secondary) is added.
5) A substrate is added to react with enzyme coupled with Ag/Ab complex.
6) Coloured product is obtained followed by measurement by a photometer.

Indirect ELISA: It is used to detect antibodies in the sample. Wells are coated with specific antigen. Two (primary & secondary) antibodies are used in this type. Its example is detection of HCV Antibodies in the patient blood.

Sandwich ELISA: It is used to detect antigen in the sample. Well surface is labeled with specific antibodies. The required antigen becomes sandwich

between two antibodies. Its example is detection of HBV surface antigen in a patient blood.

ELISA is also used in other industries in addition to diagnostics like food industry to detect food allergens in milk, walnuts, almonds and eggs and in toxicology to screen the classes of drugs. ELISA has developed its further generations with time like Microparticle Enzyme Immunoassay (MEIA), Fluorescence Polarization Immunoassay (FPIA) and Chemiluminescence Microparticle Immunoassay (CMIA) or Electro-Chemiluminescence Immunoassay (ECLIA). These are the refined forms of ELISA that uses most specific antigen and/or antibody labeled with micro-magnetic particles and detection is made by fluorescence or electric impulse.

ANNEXES

ANNEX-1 GLOSSARY

Adjuvant is a substance that increases and enhances the efficacy of any drug/vaccine.

Aerosol It is **s**uspension in air of finely dispersed solids or liquids.

Aliquot A representative portion of a sample with a known volume/quantity.

Antibiotic It is a substance that works (either kill or stop) against microorganisms especially bacteria.

Antibody It is a kind of protein produced (naturally) by immune system of the body in response of any foreign (pathogenic) entity generally termed as Antigen.

Antigen It is a substance when introduced into the body (tissue or blood) induces the formation of Antibody.

Antitoxin Any organic inorganic or biochemical molecule which is used against the toxin (bacterial product).

Bioremediation It is the use of organisms to remediate an environmental problem.

Biosafety This refers to the containment principles, technologies and practices that are put into practice to avoid/stop unintentional exposure to microbial pathogens and toxins, or their accidental release.

Biosecurity This includes the institutional and personal security measures planed to stop the loss, theft, misuse, diversion or deliberate release of microbial pathogens and toxins.

Candidiasis Infection caused by Candida albican is known as candidiasis. Candida can cause both vaginal and oral infections.

Chromosome A chromosome is an organized structure of DNA, protein, and RNA found in cells. It is a single piece of coiled DNA containing many genes, regulatory elements and other nucleotide sequences.

Conjugation It is the mating of two bacterial cells during which DNA is transferred from the donor to the recipient cell.

Contagious Disease It is a disease transmitted though physical contract ob the secretion so the effected individual

Effluent It is a liquid (or gaseous) industrial waste.

Endemic It is the permanent maintenance/presence of a disease causing agent in a particular region.

Endotoxin These are produced in both gram negative and positive bacteria. They are polysaccharide and very toxic inducing the formation of antitoxin.

Epidemic This refers to sudden outbreak of a disease (when the new numbers of cases increase in a given time period).

Epidemiology It is a corner stone of evidence based medicine. It is the study of the patterns, causes, and effects of health and disease conditions in a particular population.

Epistasis An interaction between genes in which one gene affects the expression of another.

Epitope It is able to elicit an immune response and is a localized region on the surface of an antigen.

Eukaryotes A eukaryote is an organism whose cells contain a nucleus and other structures (organelles) enclosed within membranes

Exotoxin These are present in gram negative bacteria cell wall. They are lipopolysaccharide.

Gene A hereditary unit of nucleic acid that specifies the structure of a protein or RNA molecule.

Hybridoma A hybrid cell used in production of monoclonal antibodies that is produced by fusing an antibody-producing cell (B lymphocyte) with a tumor cell.

ID50 It is the number required to cause infection in half the hosts.

Immunity It is the state of a being resistance to injury, particularly by poisons, foreign proteins and invading parasites due to the presence of antibodies.

Immunology It is the study of immunity which is a defense mechanism of the body.

Incubation Period It is the time between the start of infection and the appearance of signs and symptoms.

Infectious Dose It is the dose of a micro-organism which is required to cause disease.

LD50 It is the number of organisms required to kill half the hosts.

Lysis It is the destruction, decomposition or disintegration of any cell (blood cell or bacteria) by the action of specific agent.

Mutagenesis These are natural or artificial procedures that cause mutations in organisms; used to create mutant organisms in research and molecular biology.

Mutation It is mis-arrangement of nucleotide sequences which result in the abnormal function of transcribed protein.

Normal Flora The microbial population present on the system surface to protect from non self organisms is called normal flora.

Oncogene An activated (modified) cellular gene that causes normal cells to become cancerous.

Pandemic It is an epidemic which has infected a larger region (e.g. many countries).

Parasitism Relationship between two organisms in which one organism (the parasite) derives benefit by growing in or on another organism (the host), and in which the host organism either derives no benefit or is harmed.

Pathogenesis It is the mechanism by which a disease is caused.

Peptidoglycan It is cell wall component of gram positive bacteria chemically composed of protein and carbohydrates.

Phagocytosis It is a form of endocytosis in which phagocytes (macrophages, a kind of WBC) engulf the microorganisms, dead cells and foreign particles.

Plasma It is the yellowish liquid portion of anticoagulated blood (mixed with EDTA, Heparin, and Citrate, etc.) obtained after centrifugation. Plasma contains the Fibrinogen and other clotting factors.

Prions It is an infectious mis-folded protein and causes encephalopathies.

Protein A molecule composed of amino acids.

Protoplasm All living part of the cell is called protoplasm. Protoplasm is the living contents of a cell that is surrounded by a plasma membrane. It is a general term for the cytoplasm

Protoplast A protoplast is a plant, bacterial or fungal cell that had its cell wall completely or partially removed using either mechanical or enzymatic means.

Quality Control These identify and minimize the laboratory errors and include the steps taken to provide evidence that results are accurate.

Receptor Cell-surface protein to which molecules, such as hormones and growth factors, bind to exert their effects on the cell, or to which viruses bind to gain entry to the cell.

Retrovirus A virus that uses the enzyme reverse transcriptase to copy its RNA genome into DNA, which then integrates into the host cell genome.

Risk Management The measures to ensure that the production and handling of organisms are safe.

Selection Pressures Natural or artificial force that favors survival of one individual or group over another individual or group in the same environment or ecosystem.

Serotype It is one of the taxonomy (classification) of microorganisms that is determined by testing of the constituent antigens present on the surface of that particular microorganism. One microorganism can possess multiple serotypes at one time.

Serum It is obtained by centrifuging clotted blood and do not contain clotting factors.

Strains These are the several form of a single bacteria each having its own characteristics.

Streaking It is a procedure which is applied to isolate a pure strain from a single species of microorganism.

Syphilis It is disease caused by bacterium Treponema pallidum and transmitted through sexual route.

Tissue Culture In vitro growth of tissue cells in nutrient medium.

Toxoid A bacterial toxin which has lost its toxicity.

Transduction It is the transfer of DNA to a cell (usually bacteria) through a virus.

Transfection It is the introduction of genetic material and even proteins into the cells.

Transformation It is the genetic alteration of the bacterial cell.

Translation In molecular biology, it is the formation of proteins from ribosomes.

Transposons These are small DNA molecules that can move in and out of specific positions within the same chromosome or another chromosome of the same or different cell or plasmid. In

Vaccine It is the preparation of killed or attenuated microorganisms or their components or their products that is used to induce active immunity against a disease.

Vector A self-replicating agent (for example, a plasmid or virus) used to transfer foreign DNA into a host cell.

Virulence It is the quantitative measure of pathogencity. It can be calculated by the number of organisms required to cause disease.

Window Period It is the time period between the start of infection and when the infection can be detectable.

Xenotropic Retrovirus A retrovirus that is endogenous to a species but cannot replicate well in that species, generally because of a receptor back. Xenotropic retroviruses tend to have a wide range for replication in cell of heterologous species.

ANNEX-2 FAMOUS MICROBIOLOGISTS

Agostino Bassi (1773-1856) He discovered the causative agent of muscardine disease of silkworms which was a fungus later named as Beauveria bassiana.

Albert Jan Kluyver (1888-1956) He proposed a theory explaining the metabolism in cells (aerobic and anaerobic).

Albert Schatz (1920-2005) He discovered streptomycin (in 1943) along with Selman A. Waksman and Elizabeth Bugie Gregory. The antibiotic was isolated from Streptomyces griseus (a soil bacterium) and used for the treatment of tuberculosis.

Alexander Fleming (1881-1955) He won the Nobel Prize in Physiology or Medicine (shared with Howard Florey and Ernst Boris Chain) in 1945 for his discovery of antibiotic Penicillin from Penicillium notatum. He also discovered the enzyme lysozyme.

Alexandre Emile Jean Yersin (1863-1943) He co-discovered the bacteria (now termed Yersinia pestis) causing the bubonic plague.

Alfred Day Hershey (1908-1997) He proposed that resistant of bacteria to viruses (bacteriophage) is genetically inherited. He won the Nobel Prize in Physiology or Medicine in 1969 along with Max Ludwig Henning Delbrück and Salvador Edward Luria.

Alice Catherine Evans (1881-1975) Her major work involves the milk and cheese microbiology and identifying the causative agent for Brucellosis.

André Lwoff (1902-1994) He won the Nobel Prize in Physiology or Medicine in 1965 (with Jacques Monod and Francois Jacob) for their research on mechanisms explaining the genetic control of enzyme and virus synthesis.

Annie Chang She worked on bacterial genetics leading to advancements in genetic engineering. In 1973, she along with co-scientists proposed that plasmids operate as vectors in bacteria to maintain cloned genes.

Antonie Philips van Leeuwenhoek (1632-1723) He was the first to study micro-organisms under his home-made microscope (50-300 times magnification). He named the micro-organisms as animalcules. He also observed spermatozoa and muscle fibers under the microscope. He is referred to as 'Father of Microbiology'.

August Karl Johann Valentin Köhler (1866-1948) He is credited to develop the Kohler Illumination technique for Microscopy.

Barry James Marshall (1951-) He shared the 2005 Nobel Prize in Physiology and Medicine with John Robin Warren for discovering the bacterium Helicobacter pylori which causes most of the peptic ulcers.

Carl Richard Woese (1928-2012) He, with his co-scientist George Edward Fox, identified and classified archaebacteria (Archaea) as a new domain through phylogenetic taxonomy of ribosomal RNA (16S).

Carmen Sanchez She, in collaboration with Francois Jacob and Jacques Monod, proposed the operon model for control of bacterial gene action in 1960.

Cesar Milstein (1927-2002) He (along with Niels Kaj Jerne and Georges Jean Franz Köhler) developed the monoclonal antibodies from hybridoma cells. They won the Nobel Prize in Physiology or Medicine in 1984.

Charles Jules Henry Nicolle (1866-1936) He won the 1928 Nobel Prize in Physiology or Medicine for his work on typhus. He proposed that lice are carriers of typhus.

Charles Louis Alphonse Laveran (1845-1922) He observed the malarial parasites in blood smear and discovered the protozoa. He also worked on trypanosomiasis. He won the 1907 Nobel Prize in Physiology or Medicine.

Charles Yanofsky () He proposed, in 1964, that gene sequences and protein sequences are co-linear in bacteria. He also revealed how RNA act as a regulatory molecule in both bacterial and animal cells.

Claire M. Fraser (1955-) Her work has resulted in the sequencing of Borrelia burgdorferi, Mycoplasma genitalium, Treponema pallidum, and Chlamydia genomes.

Colin Munro MacLeod (1909-1972) He along with Oswald T. Avery and Maclyn McCarty developed the famous Avery-MacLeod-McCarty experiment in 1944. This experiment showed that DNA is the responsible for bacterial transformation.

Cornelius Bernardus van Neil (1897-1985) He contributed in the development of bacterial taxonomy. He along with Roger Y. Stanier classified cells in prokaryotes and eukaryotes in 1962. His definition of prokaryotes is still used today.

Craig Venter (1946-) He sequenced the human genome and developed the first cell with a synthetic genome. He is currently involved with the development of synthetic biological organisms.

Daniel Nathans (1928-1999) He won the 1978 Nobel Prize in Physiology or Medicine for discovering the restriction endonucleases in a bacterial model. The prize was shared with Werner Arber and Hamilton Othanel Smith.

David Baltimore (1938-) He is a virologist and won the Nobel Prize in Physiology or Medicine in 1975 for his discovery elaborating the interaction between tumor viruses and the genetic material of the cell. The prize was shared with Howard Temin and Renato Dulbecco. He discovered the enzyme reverse transcriptase.

David Perrin He, in collaboration with Francois Jacob and Jacques Monod, proposed the operon model for control of bacterial gene action in 1960.

Dmitri Ivanowski (1864-1920) He studied the Tobacco mosaic diseases and the causative agent (Tobacco Mosaic Virus).

Edward Lawrie Tatum (1909-1975) He studies the biochemistry and genetics of fungus Neurospora crassa and proposed the "one gene, one enzyme" theory. He won the Nobel Prize in 1956 along with George W. Beadle.

Edward Anthony Jenner (1749-1823) He developed the Small pox vaccine and often referred as the 'Father of Immunology'.

Elizabeth Bugie Gregory (1921-2001) She discovered streptomycin along with Selman A. Waksman and Albert Schatz in 1943. The antibiotic was isolated from Streptomyces griseus (a soil bacterium) and used for the treatment of tuberculosis.

Emil Adolf von Behring (1854-1917) He won the first Nobel Prize in Medicine in 1901. He discovered diphtheria antitoxin and developed serum techniques for diphtheria and tetanus.

Ernst August Friedrich Ruska (1906-1988) He won the Nobel Prize in Physics in 1986 for his work on electron optics and presenting the first design of electron microscope.

Ernst Boris Chain (1906-1979) He won the Nobel Prize in Physiology or Medicine (shared with Howard Florey and Ernst Boris Chain) in 1945 for his discovery of antibiotic Penicillin from Penicillium notatum.

Esther Miriam Zimmer Lederberg (1922-2006) She has done most of her research on bacterial genetics and was the first to discover a DNA virus termed lambda bacteriophage (from Escherichia coli). She also identified the bacterial fertility factor F and did pioneer work on replica plating.

F.L. Kilbourne He gave the first evidence of a zoonotic disease in 1889 and proposed that ticks carry a micro-organism, *Babesia microti,* which causes babesiosis in humans/animals.

Ferdinand Julius Cohn (1828-1898) He is considered one of fathers of modern bacteriology. He classified bacteria based on shape in 1878 which is still used

today. He was the first to show the transformation of bacterium Bacillus from vegetative from to an endospore form during environment stress.

Francis Crick (1916-2004) He discovered the molecular structure of nucleic acids in 1953 along with James Dewey Watson and Maurice Hugh Wilkins and won the Nobel Prize in Physiology or Medicine in 1962. He also proposed the "Central Dogma" of molecular biology.

Francis Reyton Rous (1879-1970) He won a 1966 Nobel Prize Physiology or Medicine for his research in identifying the role of viruses in cancer transmissions. He identified Rous sarcoma virus.

Francois Jacob (1920-2013) He won the Nobel Prize in Physiology or Medicine in 1965 (with André Lwoff and Jacques Monod) for their research on mechanisms explaining the genetic control of enzyme and virus synthesis. He is also known for his research on lac opeon (E.coli).

Françoise Barré-Sinoussi (1947-) She won the 2008 Nobel Prize in Physiology or Medicine together with Luc A. Montagnier, for identifying the human immunodeficiency virus (HIV).

Frederick Chapman Robbins (1916-2003) He won the Nobel Prize in Physiology or Medicine in 1954 for his work on viruses causing poliomyelitis. He cultured the virus on a culture media using a combination of human embryonic skin and muscle tissue. The prize was shared with John Franklin Enders and Thomas Huckle Weller. He also worked on schistosomiasis and Coxsackie viruses.

Fredrick Griffith (1879–1941) He worked extensively on molecular genetics and discovered bacterial transformation (Streptococcus pneumonia) in 1928 through Griffith's Experiment. He used in the experiment.

Fredrick Sanger (1918-) He received two Nobel Prizes; first in 1958 for protein structure and second in 1980 along with Walter Gilbert for determining the base sequences in nucleic acids.

George Edward Fox (1945-) He, with his co-scientist Carl Richard Woese, identified and classified archaebacteria (Archaea) as a new domain through phylogenetic taxonomy of ribosomal RNA (16S).

George Wells Beadle (1903-1989) He studies the biochemistry and genetics of fungus Neurospora and proposed the 'one gene, one enzyme' theory. He won the Nobel Prize in Physiology or Medicine in 1956 along with Edward Lawrie Tatum.

Georges Jean Franz Kohler (1946-1995) He (along with Niels Kaj Jerne and César Milstein) developed the monoclonal antibodies from hybridoma cells. They won the Nobel Prize in Physiology or Medicine in 1984.

Gerhard Johannes Paul Domagk (1895-1964) He won the 1939 Nobel Prize in Physiology or Medicine for his discovery of Sulfonamidochrysoidine, which was the first antibiotic made available commercially.

Girolamo Fracastoro (1478-1553) He proposed a theory that infectious diseases are caused and transmitted by spores which is now replaced by a germ theory. The name of the disease 'Syphilis' also came from his poems.

Hamilton Othanel Smith (1931-) He won the 1978 Nobel Prize in Physiology or Medicine for discovering the restriction endonucleases (type II restriction enzymes) in a bacterial model. The prize was shared with Werner Arber and Daniel Nathans. He was part of a team which sequenced the genome of Haemophilus influenzae in 1995.

Hans Christian Joachim Gram (1853-1938) He developed a staining method, called 'Gram Staining Technique', for differentiating between two major classes of bacteria, the Gram Positive and Gram Negative.

Har Gobind Khorana (1922-2011) He won the Nobel Prize in Physiology or Medicine in 1968 for his work on genetic code and investigating its role in protein synthesis. The award was shared with Robert William Holley and Marshall Nirenberg.

Harald zur Hausen (1936-) He discovered the viral causative agent of cervical cancer (papilloma viruses) that led to the development of the HPV vaccine. He won the 2008 Nobel Prize in Physiology or Medicine (shared with Françoise Barré-Sinoussi and Luc A. Montagnier).

Hendrick Jean Louis Donker He, along with Albert Jan Kluyver, proposed a theory in 1926, explaining the metabolism in cells (aerobic and anaerobic).

Herbert Boyer (1936-) He studied the genetically modified bacteria and produced synthetic insulin and growth harmone.

Holger W. Jannasch (1927-1998) He was a marine microbiologist who studied the microbial life in deep-sea environment.

Howard Florey (1898-1968) He won the Nobel Prize in Physiology or Medicine (shared with Alexander Fleming and Ernst Boris Chain) in 1945 for his discovery of antibiotic Penicillin from Penicillium notatum.

Howard Robert Horvitz (1947-) He won the Nobel Prize in Physiology or Medicine in 2002 along with John Edward Sulston and Sydney Brenner for his

work on a soil nematode Caenorhabditis elegans as a model organism to study developmental biology.

Howard Taylor Ricketts (1871-1910) He investigated the microbial cause of Rocky Mountain spotted fever and is named after him, Rickettsia rickettsii.

Ignaz Philipp Semmelweis (1818-1865) He was involved with studies on antiseptic procedures. He proposed the use of chlorinated lime solutions for puerperal fever.

Ilya Ilich Metchinkoff (1845-1916) He received the 1908 Nobel Prize in Physiology or Medicine for his research on phagocytosis.

J. Heinrich Matthaei (1929-) He worked on genetic code and revealing its role in protein synthesis.

Jacques Monod (1910-1976) He won the Nobel Prize in Physiology or Medicine in 1965 (with André Lwoff and Francois Jacob) for their research on mechanisms explaining the genetic control of enzyme and virus synthesis. He is also known for his research on lac opeon (E.coli).

James Dewey Watson (1928-) He discovered the molecular structure of nucleic acids in 1953 along with Francis Crick and Maurice Hugh Wilkins and won the Nobel Prize in Physiology or Medicine in 1962.

John Edward Sulston (1942-) He won the Nobel Prize in Physiology or Medicine in 2002 along with H. Robert Horvitz and Sydney Brenner for his work on a soil nematode Caenorhabditis elegans as a model organism to study developmental biology.

John Franklin Enders (1897-1985) He is called the 'Father of Modern Vaccines' and won the Nobel Prize in Physiology or Medicine in 1954 for his work on viruses causing poliomyelitis. He cultured the virus on a culture media using a combination of human embryonic skin and muscle tissue. The prize was shared with Thomas Huckle Weller and Frederick Chapman Robbins.

John Robin Warren (1937-) He shared the 2005 Nobel Prize in Physiology and Medicine with Barry James Marshall for discovering the bacterium Helicobacter pylori (in 1979) which causes most of the peptic ulcers.

John Snow (1813-1858) He investigated the source of a cholera outbreak in London and termed as the 'Father of Epidemiology'.

John Tyndall (1820-1893) He discovered the process of tyndallization which is a sterilization technique for food.

Joseph Lister (1827-1912) He is known as the 'Father of Antiseptic Surgery'. He introduced the use of phenol for the sterilization of medical instruments.

Joshua Lederberg (1925-2008) He studied the bacterial genetic recombination and the organization of the genetic material and won the Nobel Prize in 1958. He also did pioneer work on replica plating.

Julius Richard Petri (1852-1921) He invented the Petri dish which is widely used for microbial growth.

Julius Wagner-Jauregg (1857-1940) He won the 1927 Nobel Prize in Physiology or Medicine his discovery of the therapeutic value of malaria inoculation to treat tertiary syphilis.

Kary Banks Mullis (1944-) He won the 1993 Nobel Prize in Chemistry for his discovery of Polymerase Chain Reaction, the most common technique used in molecular microbiology.

Kiyoshi Shiga (1871-1957) He identified the causative agent of dysentery, Shigella dysenteriae named after him.

Leland Harrison Hartwell (1939-) He identified key molecular steps in the cell cycle using yeast as a model organism. He identified two important proteins, cyclin and CDK. He won the Nobel Prize in Physiology or Medicine in 2001 (shared with Paul Nurse and Richard Tim Hunt).

Louis Jablot (1645-1723) He proved biogenesis by performing experiments employing hay infusion.

Louis Pasteur (1822-1895) He is best known for a technique, pasteurization, which involves the treatment of milk and wine to prevent bacterial contamination. He identified the cause of fermentation which is due to micro-organisms growth. He also worked on vaccination against tetanus and Silk Worm Disease.

Luc Antoine Montagnier (1932-) He won the 2008 Nobel Prize in Physiology or Medicine, together with Françoise Barré-Sinoussi, for identifying the human immunodeficiency virus (HIV).

Maclyn McCarty (1911-2005) He along with Oswald T. Avery and Colin M. Macleod developed the famous Avery-MacLeod-McCarty experiment in 1944. This experiment showed that DNA is the responsible for bacterial transformation.

Margret Pittman (1901-1995) She did extensive research on pertussis which resulted in the development of whooping cough vaccination.

Marshall Warren Nirenberg (1927-2010) He won the Nobel Prize in Physiology or Medicine in 1968 for his work on genetic code and investigating its role in protein synthesis. The award was shared with Har Gobind Khorana and Robert William Holley.

Martha Cowles Chase (1927-2003) She proposed that traits are inherited through DNA and not the protein.

Martinus Beijerinck (1851-1931) He was a pioneer in agricultural and industrial microbiology and also studied the tobacco mosaic virus.

Matthew Stanely Meselson (1930-) He is a molecular microbiologist. His major work includes DNA replication and recombination.

Maurice Hugh Frederick Wilkins (1916-2004) He discovered the molecular structure of nucleic acids in 1953 along with James Dewey Watson and Francis Crick and won the Nobel Prize in Physiology or Medicine in 1962.

Max Ludwig Henning Delbruck (1906-1981) He proposed that resistant of bacteria to viruses (bacteriophage) is genetically inherited. He won the Nobel Prize in Physiology or Medicine in 1969 along with Alfred Day Hershey and Salvador Edward Luria.

Niels Kaj Jerne (1911-1994) He (along with Georges Jean Franz Kohler and César Milstein) developed the monoclonal antibodies from hybridoma cells. They won the Nobel Prize in Physiology or Medicine in 1984.

Norton David Zinder (1928-2012) He did experiments on Salmonella and discovered that bacteriophage can transfer genes from one bacterium to another.

Oswald Theodore Avery (1877-1955) He made an important discovery by experiments on bacteria that DNA is the carrier of genes in cells, regarded as one of the first molecular microbiologists. He along with *Colin* Munro *MacLeod* and Maclyn McCarty developed the famous Avery-MacLeod-McCarty experiment in 1944. This experiment showed that DNA is the responsible for bacterial transformation.

Paul Berg (1926-) He won the Nobel Prize in Chemistry in 1980. He studied the biochemistry of nucleic acids and developed the first recombinant DNA molecule from cancer-causing monkey virus SV40. His experiments were related to gene splicing.

Paul Ehrlich (1854-1915) He was one of the most outstanding microbiologists/immunologists of all times. He discovered the treatment (Arsphenamine) for syphilis and did pioneer work on diphtheria antiserum. He received a Nobel Prize in Physiology or Medicine in 1908.

Paul Nurse (1949-) He identified key molecular steps in the cell cycle using yeast as a model organism. He identified two important proteins, cyclin and CDK. He won the Nobel Prize in Physiology or Medicine in 2001 (shared with Leland Harrison Hartwell and Richard Tim Hunt).

Peter Dennis Mitchell (1920-1992) He won the 1978 Nobel Prize in Chemistry for discovering the chemiosmotic theory of ATP synthesis.

Rebecca Craighill Lancefield (1895-1981) Her major work include research on Group A Streptococci and their association with rheumatic fever. She also classified the beta-haemolytic streptococci serologically which is now termed 'Lancefield Classification'.

Richard Tim Hunt (1943-) He identified key molecular steps in the cell cycle using yeast as a model organism. He identified two important proteins, cyclin and CDK. He won the Nobel Prize in Physiology or Medicine in 2001 (shared with Leland Harrison Hartwell and Paul Nurse)

Robert Charles Gallo (1937-) He played a major role in the identification of infectious agent for AIDS, the HIV. He showed the possible role of chemokines in controlling the HIV infection. He also discovered Human herpesvirus 6.

Robert Heinrich Herman Koch (1843-1910) He identified the micro-organisms causing tuberculosis, cholera, and anthrax. He also formulated Koch's Postulates. For his research on tuberculosis, he won the Nobel Prize in 1905.

Robert Helling He worked on bacterial genetics leading to advancements in genetic engineering. In 1973, he along with co-scientists proposed that plasmids operate as vectors in bacteria to maintain cloned genes.

Robert William Holley (1922-1993) He won the Nobel Prize in Physiology or Medicine in 1968 for his work on genetic code and investigating its role in protein synthesis. The award was shared with Har Gobind Khorana and Marshall Warren Nirenberg.

Roger Yate Stanier (1916-1982) He contributed in the development of bacterial taxonomy. He along with Cornelius Bernardus van Niel classified cells in prokaryotes and eukaryotes in 1962. He also classified blue-green algae as cyanobacteria. He also co-authored a book titled The Microbial World.

Rosalind Elsie Franklin (1920-1958) She made important contributions to the discovery of DNA molecular structure. She also worked on viruses during the later part of her life including polio virus and tobacco mosaic virus.

Ruth Ella Moore (1903-1994) Her major work involves the study of enterobacteriaceae and blood groups.

Salvador Edward Luria (1912-1991) He proposed that resistant of bacteria to viruses (bacteriophage) is genetically inherited. He won the Nobel Prize in Physiology or Medicine in 1969, along with Alfred Day Hershey and Max Ludwig Henning Delbrück.

Selman Abraham Waksman (1888-1973) He discovered streptomycin (in 1943) along with Albert Schatz and Elizabeth Bugie Gregory. The antibiotic was isolated from Streptomyces griseus (a soil bacterium) and used for the treatment of tuberculosis. He won the Nobel Prize in Physiology or Medicine in 1952.

Sergei Winogradsky (1856-1953) He studied nitrifying bacteria and reported the chemoautotrophy.

Shibasaburo Kitasato (1853-1931) He co-discovered the bacteria (now termed Yersinia pestis) causing the bubonic plague. He also was the first scientist to culture the bacterium Clostridium tetani.

Stanley Benjamin Prusiner (1942-) He identified prions and received 1997 Nobel Prize for his research on investigating the cause of mad cow disease.

Stanley Norman Cohen (1935-) He worked on bacterial genetics leading to advancements in genetic engineering. His main research focused on bacterial plasmids and also worked on bacterial genetic transformation.

Sydney Brenner (1927-) He won the Nobel Prize in Physiology or Medicine in 2002 along with H. Robert Horvitz and John Sulston for his work on a soil nematode Caenorhabditis elegans as a model organism to study developmental biology.

Theobald Smith (1859-1934) He gave the first evidence of a zoonotic disease in 1989 and proposed that ticks carry a micro-organism, *Babesia microti*, which causes babesiosis in humans/animals.

Thomas Dale Brock (1926-) He discovered the bacteria Thermus aquaticus which is important for DNA amplification. The bacteria are hyperthermophile in nature.

Thomas Huckle Weller (1915-2008) He won the Nobel Prize in Physiology or Medicine in 1954 for his work on viruses causing poliomyelitis. He cultured the virus on a culture media using a combination of human embryonic skin and muscle tissue. The prize was shared with John Franklin Enders and Frederick Chapman Robbins. He also worked on schistosomiasis and Coxsackie viruses.

Walter Gilbert (1932-) He proposed a theory about existence of introns and exons and also developed a novel method for DNA sequencing. He received the 1980 Nobel Prize in Chemistry along with Frederick Sanger.

Walter Reed (1851-1902) He proposed that yellow fever is not transmitted by direct contact but by a particular mosquito species carrying yellow fever virus.

Wendell Stanley (1904-1971) He won the 1946 Nobel Prize in Chemistry and worked on tobacco mosaic disease caused by tobacco mosaic virus.

ANNEX-3 AUTHORS' PROFILE

Usman Waheed has an extensive experience in teaching graduate and undergraduate level MLT courses. He has published 20 research papers in national and international Journals besides authoring three handbooks related to Laboratory Sciences. He supervised the team formulating the National HIV testing strategy for Pakistan and currently investigating the molecular and genetic features of HIV in disease pathogenesis. A member of many professional bodies and international expert working groups, Mr. Waheed is serving on Advisory Board of American Society for Clinical Pathology and Safe Blood Transfusion Programme, Pakistan.

Asim Ansari is a Medical Laboratory Technology graduate followed by MPhil in Biotechnology. His core competencies include Laboratory/Healthcare Management, LIS/HMIS domain expert, ISO Quality Management, Infection Control and Personnel Capacity Development in Laboratory Sciences. He is a certified ISO-15189:2007 Technical Assessor by Pakistan National Accreditation Council (PNAC) and Norwegian Accreditation (NA). He is also the member of International Federation of Infection Control (IFIC) and American Society for Microbiology (ASM). He was also the co-author, with Usman Waheed, of handbooks *"Histotechniques"* & *"Serological Techniques in Immunology"* for laboratory professionals.

Anwar Ullah has an extensive hands-on working experience in a clinical microbiology laboratory. He completed his MPhil in Population Genetics and conducted his research at a reputed research institution of Pakistan. He has been employing microorganisms in several of his research studies including expression analysis. He has supervised a number of students and currently involved in elucidating neuro-genetics and developmental genetics.

Ihsan Ali is a young microbiologist with plenty of experience working in a clinical microbiology laboratory. As a microbiologist, he is involved in monitoring and assessing samples from a range of sources, using a variety of identification methods, including molecular techniques. He has investigated the molecular mechanism of uropathogenic E. coli pathogenesis and currently working on clinical bacteriology and microbial genetics.

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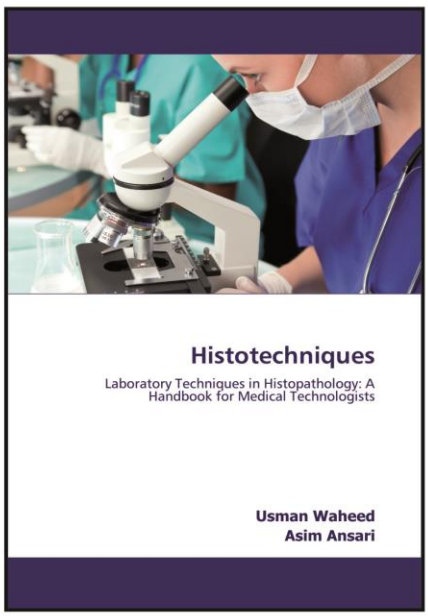

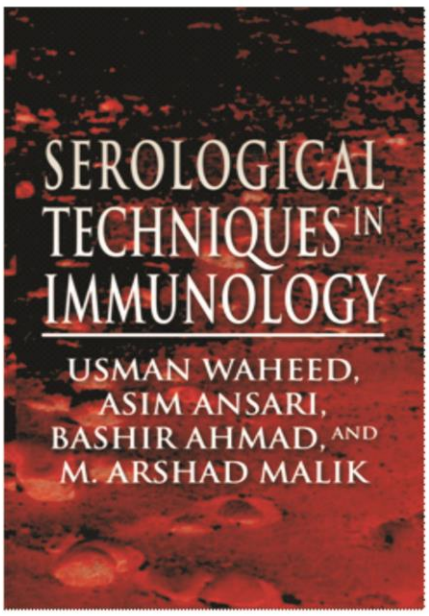

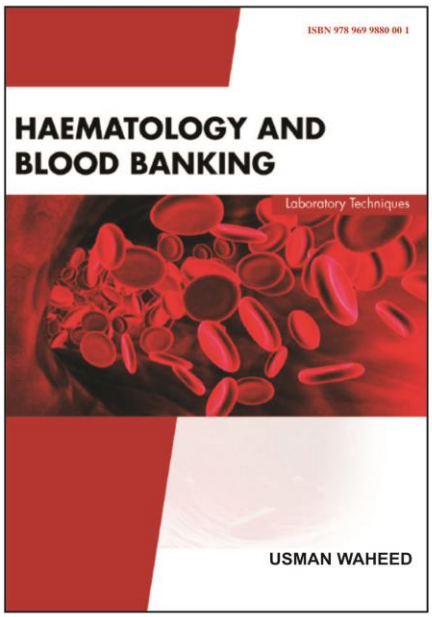

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